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# VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

*Edited by*

ROBERT S. HARRIS and KENNETH V. THIMANN

*Associate Professor of Nutritional Bio-  
chemistry, Massachusetts Institute of  
Technology, Cambridge, Mass.*

*Associate Professor of Plant Physiology  
Harvard University  
Cambridge, Mass.*

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## CONTRIBUTORS TO VOLUME III

- RACHEL BARRETT, *Harriet Lane Home, Johns Hopkins Hospital, Baltimore, Maryland*
- FLOYD S. DAFT, *Department of Physiology, National Institute of Health, Bethesda, Maryland*
- F. C. DODDS, *Courtauld Institute of Biochemistry, Middlesex Hospital, London, W.1, England*
- MILTON ELKIN, *Lederle Laboratories, Pearl River, N. Y.*
- A. BAIRD HASTINGS, *Harvard Medical School, Boston, Mass.*
- B. C. J. G. KNIGHT, *Wellcome Physiological Research Laboratories, Langley Court, Beckenham, Kent, England*
- THOMAS MOORE, *Dunn Nutritional Laboratory, Cambridge, England*
- DAVID NACHMANSOHN, *Department of Neurology, College of Physicians and Surgeons, New York, N. Y.*
- VICTOR A. NAJJAR, *Department of Pediatrics, Johns Hopkins Hospital, Baltimore, Maryland*
- W. H. SEBRELL, *Department of Physiology, National Institute of Health, Bethesda, Maryland*
- Y. SUBBAROW, *Lederle Laboratories, Pearl River, N. Y.*
- FELIX SULMAN, *Hormone Research Laboratory of the Hebrew University, Jerusalem, Palestine*
- JOSEPH WARKANY, *Childrens Hospital Research Foundation, Cincinnati, Ohio*
- BERNHARD ZONDEK, *Hormone Research Laboratory of the Hebrew University, Jerusalem, Palestine*



## Editors' Preface

The continued success of VITAMINS AND HORMONES indicates that these volumes are meeting a need for critical summaries of the progress of vitamin and hormone research.

Only those who have attempted to write a critical scientific review realize how much intense work and careful thought have entered into the preparation of the chapters contained in the present volume. The material has been gleaned from a variety of chemical, physical, biological and medical journals, then cautiously digested and carefully presented. The Editors wish to express their appreciation to each author for his contribution to the success of this publication.

Book reviews of previous volumes have offered constructive criticisms that have been helpful to the Editors in planning for future volumes of VITAMINS AND HORMONES. To some reviewers and readers it has seemed that each volume is a series of disconnected articles. Since there are many vitamins and hormones and since the research on each must be reviewed from the chemical, biological and clinical viewpoint before the subject is completely covered, the contents of any one volume must necessarily be somewhat unrelated. The subject matter of successive volumes will integrate more and more until VITAMINS AND HORMONES eventually becomes a complete reference to all active research in the vitamin and hormone field. Meanwhile, each chapter will serve as a thorough summary of progress in a small area of these fields and point the way toward which future research should be directed.

ROBERT S. HARRIS  
KENNETH V. THIMANN

August, 1945  
Cambridge, Massachusetts



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# The Interrelation of Vitamins

By THOMAS MOORE

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## I. INTRODUCTION

In discussions on the possibility of setting up standard values for the requirements of vitamins by man and animals most opinions lie between two extremes. According to one school of thought the problem presents little difficulty. On the principle that the speed of a navy is that of its slowest ship the deciding factor in determining the adequacy of any diet will, in the long run, be that vitamin which is present in the least amount in relation to the physiological requirement. The provision of extra supplies of other nutrients will not afford any compensation, and the requirement of each vitamin may therefore be considered separately as a simple question concerning that vitamin only. At the other extreme, the view is held that the requirement for one vitamin will be influenced so profoundly by the intakes of others that it is impossible to fix a minimum requirement which will remain constant when the other constituents of the diet are changed. Thus the standard value for the requirement of one particular vitamin, as estimated under experimental conditions with a basal diet which is intended to contain adequate amounts of all other vitamins, is considered to be liable to grave inaccuracy when the basal ration is replaced by an ordinary mixed diet of uncontrolled vitamin content.

The purpose of this article is to collect evidence on the "interrelation" of vitamins. This term has been widely interpreted to include not only instances in which the intake of one vitamin influences numerically the requirement of another, but also cases in which vitamins participate in related physiological or biochemical systems, or are concerned in the development and maintenance of the same tissues. Some notes on termin-



ology may not be out of place. "Interrelation" has been used in a general sense to cover all aspects of interplay between vitamins. "Association" implies that two vitamins are concerned in the same sphere of influence, whether defined physiologically, biochemically, or morphologically, but does not infer a direct chemical or physiological connection, or imply that the requirement of one is necessarily influenced by the intake of the other. "Interaction" is reserved for instances in which a direct connection, either chemical or physiological, between two vitamins has been proved. For interaction in which the requirement of one vitamin is reduced by a liberal intake of another the term "synergism" as used by Hickman (1) may be appropriate. The term "secondary deficiency" means a deficiency of vitamin which is not merely due to a low dietary intake, but which is "conditioned" or aggravated by some other factor, such as an inadequate supply of another vitamin. It is not used in the sense accepted by some workers to designate the less conspicuous of two superimposed deficiencies, for which "concurrent" or "minor" would seem to be more apt descriptions.

Since no serious attempt to deal with the interrelation of vitamins seems hitherto to have been made, the author in his search for material has been denied the usual assistance afforded by the bibliographies of previous reviews. Apologies are offered to the authors of any relevant work which has inadvertently been overlooked.

## II. THE INTERRELATION OF VITAMINS IN VITRO

It will be convenient to discuss instances of interrelations between vitamins under separate headings according to the circumstances under which they have been observed. Clearly, however, such classification will often depend on the particular aspect of the complete story which has so far come to light. An interrelation which has already been found *in vivo* may later be found *in vitro*, or *vice versa*, as indeed has already occurred in one instance. Classification is therefore based on the evidence at present available, and the distinctions drawn between different types of interrelation may not always be genuine.

### 1. Carotene, Vitamin A, Linoleic Acid, Tocopherol, and Ascorbic Acid

The potency of the tocopherols as antioxidants enables them to protect many of the readily oxidizable constituents of fats, including carotene and vitamin A. Some of the work in this field was reviewed briefly by Mason in Volume 2 of this series (1944). Golumbic (2) studied the action of tocopherols in stabilizing animal fats and related substances, and found that their antioxidant power was shared by hydroxychromans and hydroxycoumarans. Quackenbush, Cox, and Steenbock (3) found that

$\alpha$ -tocopherol protected carotene when dissolved in ethyl linolate. It may be recalled that the oxidation of carotene in purified esters, with a corresponding loss in biological activity was reported many years ago by Hume and Smedley-Maclean (4). According to Quackenbush and his colleagues, tocopherol was no better than hydroquinone as an antioxidant for carotene *in vitro*, but was much more effective in protecting the carotene, and so securing full biological activity, when the solution in ethyl linolate was administered to rats. This relatively greater power of tocopherol in protecting carotene in the alimentary tract was ascribed to its remaining with the fatty phase during digestion, whereas hydroquinone would presumably be largely separated into the aqueous phase. Recently Hove (5) extracted a lipoxidase, capable *in vitro* of oxidizing carotene in the presence of linoleic acid, from the stomach of rats, but not from their intestines. This enzyme is inhibited by  $\alpha$ -tocopherol. Hilditch (6) has found that tocopherol protects preformed vitamin A in margarine when it is exposed to air at room temperature.

Another vitamin which may be concerned in the oxidation of fats is ascorbic acid, which according to Golumbic and Mattill (7) enhances the activity of tocopherols in preventing the oxidation of certain vegetable fats. Aqueous colloidal solutions of carotene, without tocopherol, are also protected by ascorbic acid (8). It is clear, therefore, that the vitamins mentioned, and also the "essential" linoleic acid, may interact in fatty mediums either as substances liable to oxidation, or as antioxidants. It seems probable that future research will reveal other instances of interaction in fatty media, and possibly will implicate other vitamins. Already Pavcek and Shull (9) have shown that biotin is inactivated by rancid fats, from which it is protected by tocopherol. While in some cases the chemical behavior of the vitamins in fats may be related to their physiological activity, in others their reactions when exposed to oxidative influences may merely conform with those of the other constituents of the fat.

## 2. Ascorbic Acid and Riboflavin

Kon and Watson (10) found that the exposure of cows' milk to light caused a rapid destruction of ascorbic acid. Since the effective rays were found to be in the visible region, in which ascorbic acid has no absorption, it was obvious that the reaction was activated by some other substance or substances capable of absorbing visible light. Later it was found by Hopkins (11) that ascorbic acid is rapidly destroyed when exposed to sunlight in the presence of riboflavin. The riboflavin itself was converted to lumichrome and other products, which were however still capable of acting as sensitizers for the oxidation of ascorbic acid. Hand, Guthrie,

and Sharp (12) showed that the photochemical oxidation of ascorbic acid in milk was due to the presence of riboflavin and could be prevented by the removal of oxygen.

### III. THE ASSOCIATION OF VITAMINS IN FUNCTION

#### 1. *Ascorbic Acid, Vitamin P, and Capillary Resistance*

The best example of combined action of vitamins in performing a single function, if the evidence so far available may be accepted, is perhaps to be seen in the interaction of ascorbic acid and vitamin P in preventing capillary hemorrhage. The early attempts of Bentsath, Rusznyak, and Szent-Györgyi (13, 14) to demonstrate that guinea pigs needed an additional vitamin to supplement the action of ascorbic acid did not carry conviction. Zilva (15) and Moll (16) were both unable to confirm the claims of Szent-Györgyi and his colleagues, who was himself unable to repeat his experiments (17). Scarborough (18) and Zacho (19), however, confirmed the action of vitamin P, in the form of citrin or other flavone pigments, in maintaining capillary resistance. Citrin alone considerably increased capillary resistance, but the simultaneous presence of both citrin and ascorbic acid was said to be necessary for the maintenance of resistance at its normal level. Bacharach, Coates, and Middleton (20) have proposed the introduction of a biological test in which capillary resistance in guinea pigs given adequate doses of ascorbic acid was found to vary consistently with graded doses of vitamin P, which was given either as crystalline hesperidin, or as an aqueous extract of orange peel and pulp. An experiment in the converse direction, to find whether capillary resistance is correlated with the ascorbic acid intake when vitamin P is controlled at an adequate level, seems highly desirable.

#### 2. *Vitamin A, Riboflavin, and Ascorbic Acid in Dark Adaptation*

The preservation of normal powers of dark adaptation is another function in which, according to some workers, more than one vitamin is concerned. Kimble and Gordon (21) found that most individuals in their experiments who had poor dark adaptation and a low level of vitamin A in the blood could be brought into the normal ranges for both these criteria by dosing with vitamin A. Other individuals, however, failed to show the usual response. This led to the belief that other factors must be involved in the utilization of vitamin A for the synthesis of visual purple, and both riboflavin and ascorbic acid were tried as supplements to the action of vitamin A. Several otherwise normal subjects with poor dark adaptation and low blood vitamin A were given 200,000 I.U. of vitamin A daily as halibut liver oil, but failed to respond. It is perhaps rather difficult in the

absence of fever or liver disease to understand why the blood vitamin A remained low when such massive doses of halibut liver oil were given, unless the normal post-absorptive rise was intentionally avoided by taking blood samples shortly before administering the daily dose. According to Kimble and Gordon, however, normal values for blood vitamin A and normal dark adaptation were usually restored at once in these cases by giving 1 mg. of pure riboflavin daily in addition to vitamin A. In other subjects who did not benefit from this treatment, satisfactory improvement was obtained by giving ascorbic acid.

The possibility that ascorbic acid may be concerned in dark adaptation has also been suggested by Stewart (22). He reports that in attempts to improve dark adaptation by additions of food to the diet, oranges produced much greater improvement than did eggs or tomatoes, when given in amounts which provided equal quantities of carotene. Pure ascorbic acid, in daily doses of 150 mg., produced as great an improvement in dark adaptation as did daily doses of 24,000 I.U. of vitamin A. Examination of dietary histories appeared to indicate that good adaptation was invariably shown by subjects with good intake of vitamin A and ascorbic acid, and poor adaptation by those with low intakes, although there was a large intermediate group impossible to interpret.

#### IV. THE ASSOCIATION OF VITAMINS IN RELATED BIOCHEMICAL SYSTEMS

##### *1. Vitamin A, Riboflavin, and Visual Purple*

Morton (23) has given various grounds for criticizing Wald's theory (see Wald in Volume 1 of this series) that visual purple is a complex made up of vitamin A and protein. Thus he points out that we have no precedent from which to expect that the colorless vitamin should form a rose-colored complex, or should give products which are abnormally photo-sensitive. Moreover it is difficult to understand why the photo-degradation products of vitamin A, transient orange and indicator yellow, should be sensitive to pH. Morton recalls that Adler and Euler (24) reported that a high concentration of riboflavin is present in the retina of fish in free, rather than phosphorylated form. Since free riboflavin is present in no other part of the body they have suggested that it may not function as a redox catalyst in the eye, but may take part in the visual processes by virtue of its photochemical properties, such as fluorescence and sensitivity to light.

Although neither free nor phosphorylated riboflavin has an absorption spectrum similar to that of visual purple, Morton further recalls that intense red intermediate products are formed when riboflavin, or related compounds such as lumiflavin, are reduced in strongly acid solution (25, 26). A red compound, presumably a flavin-pyridine-nucleotide, is moreover

produced when yellow enzyme is reduced in the presence of excess of triphosphopyridine-nucleotide (27), and is considered by Morton to be the "closest physiologically plausible analog" to visual purple which is so far known. The rôle in dark adaptation assigned by Morton to vitamin A is to effect the reduction of a yellow riboflavin complex to form visual purple. In this process the vitamin A itself is assumed to be oxidized to the corresponding aldehyde. At this point, Morton and Goodwin (28) have strengthened the chain of conjectures with a valuable addition to the experimental evidence. Vitamin A aldehyde, prepared by the oxidation of the vitamin with potassium permanganate, has been shown to be identical with retinene, as observed in extracts of light adapted retinae, in regard to its spectroscopic absorption maxima, both in the ultra violet region and in the antimony trichloride reaction. While much more experimental evidence is necessary before a decision can be made whether this new theory of the interaction of two vitamins should supplant the older single vitamin theory of Wald, it will in any case serve a useful purpose in focusing attention more sharply on the chemical aspects of the problem, and the difficulties to be faced. The new theory, of course, agrees well with Kimble and Gordon's conclusion that riboflavin therapy may improve dark adaptation. The intervention of ascorbic acid, which has also been mentioned in regard to dark adaptation, in a photosensitive system for the reduction of riboflavin would not be surprising in view of the observation of Hopkins that these vitamins interact *in vitro*.

## 2. Thiamine, Niacinamide, and Riboflavin in Carbohydrate Metabolism

The actions of these three vitamins are associated in so far as they are all known to be concerned in the intermediate metabolism of carbohydrate. The possibility that thiamine might be concerned in carbohydrate metabolism of animals was investigated by Peters and his colleagues as early as 1930. At first it was thought that the vitamin was concerned in the oxidation of lactate (29) but later it appeared that the vitamin was connected in some way with the breakdown of carbohydrate at the pyruvic acid stage (30, 31, 32). Later, Lohmann and Schuster (33) proved that cocarboxylase, the detachable prosthetic group of the decarboxylating enzyme carboxylase, consists of thiamine combined with pyrophosphoric acid. The presence of cocarboxylase in yeast, liver, kidney, and other biological material indicates that thiamine is essential for the carbohydrate metabolism of many types of tissues. Both riboflavin and niacinamide are also known to participate in carbohydrate metabolism as essential components of dehydrogenase systems. Since the discovery of the old "yellow enzyme" of Warburg and Christian (34, 35, 36, 37), numerous other flavoproteins have been found in which riboflavin constitutes part of the mono- or

di-nucleotides which act as the prosthetic group of these enzymes. Niacinamide also fills familiar roles as a constituent of di-phosphopyridine nucleotide, the coenzyme 1 of Euler (38), and of tri-phosphopyridine nucleotide, the coenzyme 2 of Warburg (35, 39, 40).

While there is thus ample evidence that these three members of the vitamin B complex all participate in carbohydrate metabolism, it would be an obvious fallacy to conclude that their activities are necessarily confined only to this sphere. Thus the coenzyme concerned in the oxidative deamination of  $\alpha$ -amino acids (41) has been shown by Warburg and Christian (42) to be a dinucleotide containing riboflavin. Ratner, Noeito, and Green (43) have recently discovered an oxidase for glycine which is also a flavoprotein.

### *3. The Interaction of Thiamine and Riboflavin as Studied in the Living Animal*

In addition to the association between the roles of thiamine and riboflavin which has been demonstrated in the study of enzyme systems or tissue preparations *in vitro*, we have some knowledge of the interplay of these vitamins in the living animal. Sure and Ford (44) have found that in rats deficient in thiamine the riboflavin content of the tissues was slightly decreased, while the excretion of riboflavin in the urine was much increased, particularly in chronic deficiency (45). This suggests a poor assimilation of riboflavin. On the other hand, deficiency of riboflavin caused a slight decrease of thiamine in the tissues, although its excretion in the urine was not altered. Approaching the problem from a slightly different angle, Supplee, Jensen, Bender, and Kahlenberg (46) found that deficiency of thiamine interfered with the mobilization of riboflavin in the liver which normally occurs during the digestion and assimilation of food. This mobilization was also affected in deficiency of pantothenic acid, but not of pyridoxin. According to Singher and his colleagues (47), however, deficiency of thiamine caused an increase of riboflavin in the liver, while conversely in riboflavin deficiency the thiamine content of this organ is increased. Deficiencies of other vitamins, whether of pyridoxin, pantothenic acid, or biotin were found to have no effect on the hepatic thiamine and riboflavin. While, therefore, opinions seem to differ on one point, *i.e.*, the effect of thiamine deficiency on the riboflavin content of the liver, there seems to be agreement that the metabolism and storage of these two vitamins are to some degree interrelated.

### *4. Riboflavin, Niacin, and Protein Metabolism*

It may be recalled that early work by Hartwell (48) and by Reader and Drummond (49), before the recognition of the multiplicity of the vitamin B complex, showed that when the protein content of the basal diet was

increased it was necessary also to raise the vitamin B allowance in order to maintain a good rate of growth. More recent investigations (50, 51) have suggested that riboflavin is necessary for the assimilation of protein, and its resynthesis into tissue proteins. In this role its action appears to be linked with that of niacin. Sarett, Klein, and Perlzweig (52) found that the urinary excretion of both riboflavin and niacin by dogs varied in an inverse relation to the protein intake, and concluded that they are associated together in protein metabolism. The site of the interaction is apparently the liver. Thus Sarett and Perlzweig (53) found that when groups of rats were given diets high and low in the vitamin B complex, or high and low in protein, little difference could be detected between the riboflavin and niacin content of the carcasses in the four groups. In the liver, however, riboflavin and niacin were increased by high protein, but were unaffected by the allowance of vitamin B complex. In contrast, the thiamine content of both the carcass and liver varied directly as the intake of vitamin B complex, and was independent of the protein intake.

#### *5. The Vitamin B Complex, Choline, Ascorbic Acid, and Vitamin A*

There is evidence, therefore, from different angles to suggest that riboflavin and niacin are concerned in the metabolism of both carbohydrates and proteins. Numerous intricacies in the overlapping of functions of these vitamins, and of others, will doubtless continue to be revealed as research develops. Already Griffith and Mulford (54) have claimed that niacin, but not other vitamins, alleviates the severity of the symptoms of choline deficiency in rats. There is also evidence that ascorbic acid status in the rat may be affected by deficiency of thiamine and other vitamins. Ray, György, and Harris (55) found that the indophenol-reducing capacity of the eye-lens was diminished by deficiency of the vitamin B complex, but were unable to trace the cause of this abnormality to the absence of any individual member of the complex known at that time. According to Kodieck and Joachim (56) the ascorbic acid content of the lens, and of certain other organs, was reduced in animals which were deprived of thiamine, but which were given the other members of the vitamin B complex as autolyzed yeast. The reduction in thiamine deficiency was much greater than in simple starvation. Sure, Theis, and Harrelson (57) found slight reductions in the ascorbic acid contents of most of the organs, not only in deficiency of thiamine, but also in deficiency of riboflavin and of vitamin A. In deficiency of pyridoxin, however, no noteworthy change was found.

#### *V. THE ASSOCIATION OF VITAMINS WITH THE DEVELOPMENT AND MAINTENANCE OF TISSUES*

If the widest possible view is taken of the effect of vitamins we may conclude that all tissues are ultimately affected to some degree by all

vitamins. Thus absence of any vitamin will eventually affect the rate of growth of the whole organism, and failure in growth will in turn influence the normal development of all parts of the body. It is clear, however, that certain tissues are more seriously affected by the absence of some vitamins than of others. The purpose of this section is to give examples of specialized tissues which are liable to serious injury through deficiency of more than one vitamin.

### *1. Vitamin A, Ascorbic Acid, Vitamin D, and Vitamin E in the Formation of Bones and Teeth*

Concentration of attention on the important role of vitamin D in the calcification of bone may lead to neglect in the study of the scarcely less important parts played by other vitamins. The characteristic skeletal lesions in infantile scurvy have, of course, been recognized for centuries. In the young child, the growing ends of the bone are most affected. Thus radiographs of the limbs in human infantile scurvy (58) often show detachment of the epiphysis from shafts of bone, or fractures near the end of the shaft. Recently, Mellanby (59) has discovered that vitamin A is also concerned in bone formation. Deficiency of this vitamin in puppies resulted in a disorganization of the activities of the osteoblasts and osteoclasts, with the result that many bones became cancellous and overgrown, with loss of their fine molding and outline. Moore and Sykes (60) have made similar observations on the bones of calves deficient in vitamin A. We have clear proof, therefore, that vitamin A, ascorbic acid and vitamin D are all essential for bone formation. Possibly a minor rôle may be played by vitamin E, either directly or through its influence on vitamin A metabolism or on the pituitary gland. Thus Barrie (61) reported that deficiency of vitamin E in rats leads to imperfect calcification of the skull, which she associates with marked degranulation of the anterior pituitary. Recently, Weissberger and Harris (62) have found, by means of experiments with labeled radioactive phosphorus, that the phosphorus "turnover" of the bones is increased in rats which have been deprived of vitamin E, or have been given excessively large amounts.

It is evident that these same vitamins are concerned in the development of teeth. The value of vitamin D in dentition has been the particular study of May Mellanby (122), who has recently suggested that the reduction in the incidence of caries in London school children is due to improved nutrition, including the increased use of vitamin D in margarine. An elegant demonstration of the well-known effect of ascorbic acid deficiencies on teeth in experimental scurvy was afforded by the experiments of Fish and Harris (63) who, by giving guinea pigs diets adequate or deficient in ascorbic acid for alternate periods, were able to produce corresponding bands of normal and degenerate structure in the incisor teeth. The effect



of vitamin A deficiency on the teeth of the rat has been studied by Wolbach and Howe (64) and others. The rich brown or orange color which distinguishes the outer fibrous layer of the anterior surfaces of the incisors is lost, and the teeth often become deformed and overgrown. T. Moore (65) has recently shown that similar depigmentation and deformation occur in rats which have adequate vitamin A reserves, but which are deficient in vitamin E. Since dental depigmentation also occurs in fluorosis (66) and in magnesium deficiency (67) it is obvious that a similar lesion may result from various dietary abnormalities, which may or may not be interrelated.

## *2. Nerve Degeneration in Vitamin Deficiencies*

Nervous incoordination is a symptom common to deficiency of several vitamins, although structural damage to the nerves, as opposed to functional derangement, appears to be caused more readily by deficiency of some vitamins than of others. The classical opisthotonus of pigeons in thiamine deficiency was one of the first signs of dietary deficiency to receive recognition. Kon and Drummond (68), however, could trace no relation between the acute nervous symptoms and degenerative changes in the nerves, which were no worse than in starved pigeons which were not suffering from vitamin B deficiency. Similarly in rats which had been subjected by Chick, Sadr, and Worden (69) to a diet deficient in vitamin B<sub>6</sub> until they had become liable to the frequent occurrence of epileptiform fits, no degeneration of the nervous system could be detected by histological methods. The flaccid palsy of the hind quarters observed by Chick and her colleagues (70) in pigs deprived of filtrate factor (pantothenic acid?) did not however respond to treatment even when the general condition of the animal improved, and permanent injury to the nerves must be inferred. Nerve degeneration also occurs both in advanced deficiency of vitamin A and of vitamin E, although other lesions involved are very different in these two deficiencies. Thus in the absence of vitamin A, injury to the nerves is associated with overgrowth of bone (59), while in the absence of vitamin E the accompanying lesion is muscular dystrophy (71). Any distinction of vitamins according to whether or not their absence causes nervous degeneration must not, however, be too rigid. Much may depend upon whether the deficiency of the vitamin is acute or chronic. Thus lesions of the nerves are found in human beri-beri, and in experimental animals which are given a diet low in thiamine over a prolonged period (72).

## *3. Skin Lesions in Vitamin Deficiencies*

Injury to the skin occurs in deficiency of many vitamins. In some instances the lesions produced are highly characteristic, as in pellagra

when fully developed in the human, and we have no evidence that they can be caused by deficiency of any vitamin except that specifically concerned. Sometimes, however, closely similar abnormalities may be found in deficiencies of different vitamins. Although hyperkeratosis is usually considered to be characteristic of deficiency of vitamin A (73, 74, 75, 76, 77, 78), D. G. Moore (79) has reported that similar lesions are found in pellagra-like conditions, while Wiltshire (80) and Fox (81) have reported that hyperkeratosis is an early sign of scurvy. More direct evidence of the association of nutrients in preserving the normality of the skin has been advanced by Birch and György (82) who have made the interesting observation that the skin lesions in deficiency of vitamin B<sub>6</sub> closely resemble those seen in deficiency of essential fatty acids. They suggested that fats may have a sparing action on vitamin B<sub>6</sub>. As the result of further experiments, Birch (83) suggested that vitamin B<sub>6</sub> is connected with the utilization of unsaturated fatty acids.

## VI. THE INFLUENCE OF FATS ON THE RELATIVE REQUIREMENTS OF VITAMINS

### 1. *Thiamine, Vitamin D and Vitamin E, and the Quantity of Fat in the Diet*

The quantity of fat present in the diet may affect considerably the requirement of vitamins. Moreover, since the need for one vitamin may be raised by increasing the intake of fat, while that for another is lowered, it seems probable that a balanced intake of vitamins which is appropriate when the diet is low in fat may become unbalanced when the diet is high in fat, or *vice versa*. Thus Evans and Lepkovsky (84) and others (85) found that a diet high in fat reduced the requirement for thiamine, and Booth, Henry, and Kon (86) found that certain fatty acids have a slight antirachitic value, thus reducing the requirement for vitamin D. Conversely, Gottlieb, Quackenbush, and Steenbock (87) have shown that a diet rich in fat increases the requirement for vitamin E.

### 2. *The Influence of Marine Fats*

The above evidence indicates that the quantity of fat in the diet has an important influence in deciding the relative amounts of vitamins which will be required. The situation is further complicated by the influence of the quality of the fat concerned, which will vary in its action according to its nature, including the degree of unsaturation and other chemical properties, and the degree of freshness. The unsaturated acids of marine fats are often injurious when given in large amounts to rats (88) or in comparatively small amounts to herbivora (89). In rats, Yoshida (90) found that the deleterious action of marine fatty acids could be counteracted

by an increased allowance of riboflavin. In rabbits, guinea pigs, and calves, however, vitamin E has been found most effective in counteracting the toxicity of marine oils. The tendency of these oils to cause muscular degeneration, and the value of vitamin E in preventing this injury, were well known before Gottlieb, Quackenbush, and Steenbock (87) found that fats in general increase the requirement for vitamin E. Thus Madsen (91) had found that cotton-seed oil, a rich source of vitamin E, was effective in preventing dystrophy in rabbits and guinea pigs, but that its beneficial action was lost if cod-liver oil was included in the diet. In the experiments of Morris (92), however,  $\alpha$ -tocopherol prevented dystrophy in rabbits even when cod-liver oil was given. Mackenzie, Mackenzie, and McCollum (93) have probably gone a long way towards reconciling these and other conflicting results by finding that tocopherol will prevent muscular dystrophy induced in rabbits by cod-liver oil provided it is given separately from the oil. If tocopherol and cod-liver oil are ingested within a few minutes of each other the protective action of the former is lost. The marine oils, therefore, seem capable of destroying vitamin E in the alimentary tract. In this and probably in other ways they make demands on the vitamin E reserves which are heavier than those of fats in general. If all the foregoing evidence can be accepted we must infer that the relative amounts of thiamine, vitamin D, vitamin E, and riboflavin needed will depend not only on the quantity, but also on the quality of fat in the diet, and even on the procedure in feeding. The converse effect, *i.e.*, the relation between vitamins and the formation of fats, does not fall within the scope of this review, but has been discussed by McHenry and Cornett in Volume 2 of this series.

## VII. THE SYNERGISM BETWEEN VITAMIN A AND VITAMIN E

This synergism may be worth consideration under a separate heading, since it has been studied more intensively and directly than any other instance of interaction. Two distinct groups of experiments have been carried out in proving the importance of vitamin E in promoting the efficient utilization of vitamin A.

### *1. The Defective Storage of Vitamin A in Animals Deficient in Vitamin E*

Moore, Martin, and Rajogopal (94) first noticed that the vitamin A reserves of the livers of rats which had been kept on a diet deficient in vitamin E, but high in vitamin A (ca. 150 I.U. daily) were much lower than the reserves of similar animals which had received the same allowance of vitamin A together with supplements of wheat-germ oil concentrate. In some experiments the differences were as much as tenfold. Moore (95) subsequently observed the same effect when synthetic  $\alpha$ -tocopherol was

substituted for the wheat-germ oil concentrate. He found that the effect of vitamin E on the storage of vitamin A in rats was much less decided when the vitamin A was given in the form of carotene. Bacharach (96) examined the liver reserves of rats given a diet with and without vitamin E for relatively short periods. Under these altered experimental conditions the difference found between the two groups was much smaller than in Moore's experiments, but it was in the same direction, and was held to be statistically significant.

A variation of the original procedure was next adopted by Davies and Moore (97). Young rats, having high reserves of vitamin A, were restricted to a basal diet deficient in both vitamins A and E, and the rate of disappearance of vitamin A was studied with and without the addition of supplements of  $\alpha$ -tocopherol. Although the vitamin A reserves of animals killed at intervals decreased in both groups, the decrease was much more rapid in the absence than in the presence of tocopherol. When after prolonged restriction to the basal diet vitamin A had disappeared from the livers of the rats without vitamin E, the animals given tocopherol still had substantial reserves. The deficiency of vitamin A in the rats without vitamin E could therefore under the circumstances of this experiment be considered as "secondary" to or "conditioned" by the vitamin E deficiency. The rats deficient in vitamin E also developed the depigmentation of the incisor teeth which had previously been observed in deficiency of vitamin A. This was at first held to provide further evidence of a secondary deficiency of vitamin A, but in later experiments pigmentation was found in rats deficient in vitamin E even when they had adequate reserves of vitamin A (65).

## *2. The Effect of Vitamin E on the Growth Responses of Animals given Small Doses of Carotene or Vitamin A*

The experiments of Hickman and his colleagues (1, 98, 99, 100) differed from those of Moore in several ways: (1) the doses of vitamin A and carotene chosen were much lower, being about the levels used in routine biological tests; (2) the animal were not kept on the basal diet long enough to develop severe deficiency of vitamin E; (3) measurement of growth responses was used instead of estimation of vitamin A reserves as an indication of synergism. The phenomenon was therefore viewed from a fresh angle. In extensive experiments with rats, Hickman, *et al.* found that vitamin E, as mixed tocopherols, increased the growth-promoting power of vitamin A alcohol, vitamin A acetate, or cod-liver oil. Thus in two groups of rats which were each given about 2 I.U. of vitamin A alcohol per animal daily the mean growth rate was about twice as great when a daily supplement of 0.3 mg. of tocopherol was given than it was without this supple-

ment. Tocopherol also increased the time taken to deplete young rats of vitamin A, and also their survival time at the end of the experiment after dosing with vitamin A had been stopped. High doses of more than 1.5 mg. of tocopherol were less effective than smaller doses in increasing growth. When small doses of carotene up to 1.7 I.U. daily were given, instead of vitamin A, increased growth was observed with doses of up to 0.5 mg. of tocopherol, but larger doses of tocopherol sometimes actually depressed growth. With large doses of carotene, growth was little affected by the tocopherol intake.

Hickman and his colleagues also found that certain other antioxidants besides the tocopherols shared their ability to cause increased growth in conjunction with vitamin A or carotene. These included lauryl hydroquinone, ascorbic acid, and palmityl ascorbic acid, but hydroquinone and *p*-aminobenzoic acid were ineffective. Combinations of different "co-vitamins" were sometimes more effective than the single substances. Thus a mixture of tocopherol and palmityl ascorbic acid caused more rapid growth than either substance given singly. The rôle of tocopherol, in common with that of the other antioxidants, is assumed to be the protection of carotene and vitamin A in the intestinal tract. This implies a general extension of the conclusions reached by Quackenbush and his colleagues in the particular instance of carotene in ethyl linolate, referred to on page 3. In agreement with the theory that the action of tocopherol is exerted in the intestinal tract, Hickman found that in one human subject the fecal excretion of carotene was increased when tocopherol was given.

Recently, Guggenheim and Hock (101) have confirmed that tocopherol both increases the storage of vitamin A in the liver, and increases the stability of carotene in the intestinal tract. In their experiments, small amounts of vitamin A or carotene were given for periods of a few days to rats which had been depleted of vitamin A. The minute reserves of vitamin which were accumulated varied according to the tocopherol intake, irrespective of whether it was given as the pure compound, or combined with the carotene supplements as a natural vegetable material. Thus when a fixed amount of carotene was given as lettuce, which is rich in tocopherol, the amount of vitamin A stored was much greater than when the carotene was given as carrot, or some other vegetable less rich in tocopherol. With doses of pure carotene in oil, the addition of tocopherol not only greatly increased the storage of vitamin but also caused a marked increase in the percentage of the ingested carotene which was excreted in the feces. The synergistic action of tocopherol, therefore, presents an acute problem to investigators who attempt to assess the efficiency of utilization of carotene from measurements of its disappearance during passage through intestinal tract. While the role of tocopherol as an intestinal antioxidant has been

proved beyond reasonable doubt, however, it must not be concluded that this is the whole story. The experiments of Davies and Moore, mentioned above, indicate that reserves of vitamin A already laid down in the liver are affected by subsequent dosing with tocopherol. This suggests that the synergistic action is also exerted in the blood stream or tissues.

#### VIII. THE EFFECT OF MASSIVE DOSING WITH ONE VITAMIN ON THE REQUIREMENT OF OTHERS

The effects of massive dosing with one vitamin on the subject's requirements for others may be conveniently reviewed under different sub-headings, according to whether the massive dosing is in itself harmless or toxic. In the first case liberal but harmless dosing with one vitamin, when not "balanced" by the concurrent administration of other vitamins, may precipitate the appearance of symptoms of deficiency of another vitamin. This may be due to the promotion of rapid preliminary growth, which will increase the demands for other vitamins. In the second case we have to discuss the possibility that the ill effects of toxic overdosage with one vitamin may be alleviated by liberal dosing with another.

##### *1. Unbalanced Dosing*

Scandinavian workers (102, 103, 104) have claimed that when human subjects suffering from multiple dietary deficiencies are dosed with thiamine alone the symptoms of niacin deficiency are developed. Similarly, Sydenstricker (105) has reported that when niacin alone is given to pellagrins certain signs of their disease, presumably due to deficiency in other factors, are intensified. An attempt by Klopp, Abels, and Rhoads (106) to produce riboflavin deficiency in man by giving large doses of thiamine was however unsuccessful, no more than a transitory rise in the urinary excretion of riboflavin being observed.

Experiments with animals have indicated that unbalanced dosing with moderate superfluity of certain vitamins has little effect. Bruce and Phillips (107) found that the giving of excess of vitamin A, thiamine and ascorbic acid had no effect on results in estimations of vitamin D, while an excess of vitamin D did not influence the assay of vitamin A. Collett and Eriksen (108) found that moderate superfluity of vitamins A and D had no antagonistic action towards ascorbic acid.

##### *2. The Alleviation of Toxic Overdosing*

It has long been known that rich sources of vitamins A and D may be toxic when given in great excess, and that benefit may sometimes be derived by giving liberal allowances of water-soluble vitamins. More than 20 years ago, Hopkins (109) mentioned that excess of cod-liver oil was injurious

to rats unless their allowance of yeast extract was raised. The problem is complicated since subsequent research has shown not only that vitamin A (110) and vitamin D (111) are each toxic in the pure form, but that they may be accompanied in their unsaponifiable concentrates by toxic impurities, and that even the fatty acids of marine oils may be injurious (88). In addition, early work suggested that isoamylamine and choline are responsible for the toxicity of some cod-liver oils, and that they produce symptoms similar to vitamin B deficiency, which may be prevented by an increased allowance of yeast (112). As antidotes for these various toxic substances a list of vitamins has been reported which is hardly less numerous. Not only the vitamin B complex, but ascorbic acid and the opposite member of the vitamin A-D partnership have been claimed to have beneficial effects.

### *3. Hypervitaminosis A Treated with Ascorbic Acid, Vitamin D, and Thiamine*

Mouriquand and his colleagues (113, 114) made early claims that excess of cod-liver oil interferes with the cure of scurvy in guinea pigs, and this observation was confirmed fifteen years later by Collett and Eriksen (108). It is not clear, however, whether the interference was due to vitamin A. Recently, Vedder and Rosenberg (115) examined the injuries produced by giving excessive amounts of jew-fish liver oil in rats. This oil is a rich source of vitamin A, which is believed by these authors to be associated with toxic impurities. The hemorrhages and skeletal fractures caused by this oil bore a remarkable resemblance to those seen in human or experimental scurvy. The urinary excretion of ascorbic acid was reduced, and it was found that the ill effects of jew-fish liver oil could be almost completely counteracted by giving large doses of ascorbic acid. Moore and Wang (110) have recently observed internal hemorrhages, differing from the diffuse types previously described, in rats given great excess of pure vitamin A acetate. They agree that the combination of bone fractures with hemorrhages is reminiscent of scurvy, but have failed to confirm either the reduced urinary excretion of ascorbic acid, or its beneficial action when given in large doses (116). Recalling the suggestion of Thoenes (117) that vitamins A and D are physiologically antagonistic, Vedder and Rosenberg (115) also tested the protective value of calciferol, and found that in appropriate dosage it shielded their animals at least partially from injury by jew-fish liver oil. Since in hypervitaminosis A the bones become soft and fragile, while in hypervitaminosis D they are subject to excessive calcification, it is possible that the two pathological processes may in a general way tend to cancel out. Moore and Wang (110), however, were again unsuccessful in an attempt to repeat this experiment, although much may depend on choosing the correct dose of calciferol. In early experiments, Harris and

Moore (118) found that the reduction in growth rate caused by giving excess of a crude vitamin A concentrate could be restored by increasing the allowance of the vitamin B complex. It is uncertain, however, whether vitamin A was the only toxic constituent of the concentrate, while no information is available as to which member of the vitamin B complex was involved.

#### *4. Hypervitaminosis D Treated with Vitamin A and Thiamine*

Gross-Selbeck (119) has reported that liberal dosing with vitamin A affords protection against injury through excess of vitamin D. According to Light, Miller, and Fry (120), the acute symptoms of overdosing with vitamin D could be counteracted at some levels by an increased allowance of yeast. Harris and Moore (121) found no improvement in rats given excess of vitamin D when their allowance of yeast extract was raised, but slight benefit resulted from combined treatment with yeast extract, wheat-germ extract, and lemon juice.

### IX. CONCLUSIONS

It would perhaps be unduly sanguine to trust that every item of the experimental evidence in favor of the foregoing examples of interrelation between vitamins would bear the test of repetition. Even greater faith would be necessary to accept each single author's interpretation of his observations as being inevitably correct. In many instances, however, a weight of evidence obtained by several investigators seems to place beyond reasonable doubt the ability of one vitamin to influence the behavior or action of another.

Any attempt to co-ordinate, rather than to collect together, the examples of interrelation so far reported might at the present stage only make confusion worse confused. If we accept the evidence now available at its face value, however, we must infer that interplay between vitamins is extensive and complicated. The various rôles of riboflavin may be taken as affording a good illustration of the complexity of the problem: (1) We are told that this vitamin reacts with ascorbic acid *in vitro* under the influence of light. (2) It is present in the retina where it possibly interacts with vitamin A in the formation of visual purple, and on separate evidence is concerned with vitamin A and ascorbic acid in dark adaptation. (3) It is associated with thiamine and niacinamide in oxidative mechanisms for carbohydrates. (4) Possibly it takes part with niacinamide in protein metabolism. (5) There is a suggestion that it has some correlation with synthesis or metabolism of ascorbic acid. (6) Finally it is said to prevent the injurious effect of marine fatty acids, which are in turn antagonistic towards vitamin E. Such a "diversity of interests" may be claimed for



several other vitamins, which leads, at least on paper, to a complicated network in which almost any two factors which have not been proved to interact directly may be assumed to influence each other through their relations with a third factor. For example, we have no evidence of direct interaction between thiamine and vitamin E, but if thiamine is "spared" by raising the proportion of fat in the diet the requirement of vitamin E will correspondingly be increased, since it is influenced in the opposite direction. To add further to the complexity of the picture we may recall that drugs of the sulfonamide class demand increased intakes of certain vitamins, although this is a matter beyond the scope of the present review. This subject is treated elsewhere in this volume.

It should be an aim of future research, therefore, to separate those instances in which there is a direct interaction between vitamins, of either theoretical or practical interest, from the less important instances in which the influence of the first vitamin on the second is merely one out of many of its effects on metabolism as a whole. An interaction strictly confined to two vitamins, in which the requirement or the metabolism of one is considerably affected by the intake of the other, is obviously of greater interest than a less specific interrelation, in which the requirement of one vitamin is only slightly affected by the intake of any of several other vitamins.

If in the main the evidence in favor of the interaction of vitamins is acceptable, there would appear to be no reason to deny that the requirement of the subject for one vitamin will be materially affected by his intake of others. In considering the adequacy of natural diets, moreover, we must also allow for the effects of certain other nutrients on vitamin requirement. Thus the consumption of unboiled egg white containing avidin will increase the demand for biotin, while the requirement for vitamin D will be influenced *inter alia* by the relative and absolute intakes of calcium, phosphorus, and phytic acid. While these difficulties in arriving at an accurate assessment of vitamin requirements should be fully recognized, however, the adoption of a hypercritical attitude towards attempts to standardize requirements would only delay progress. Current estimates of requirements should be accepted and applied on the understanding that finality has not yet been reached. Future research should aim at the compilation of more comprehensive tables, in which provision can be made to allow for the effect of such interactions between vitamins, or other nutrients, as are found to have practical importance.

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# The Synthesis of B Vitamins by Intestinal Bacteria

By VICTOR A. NAJJAR AND RACHEL BARRETT

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## I. INTRODUCTION

The term "vitamin" was originally applied to accessory organic food factors that could not be synthesized by the body. In recent years, however, it has become clear that the line between what can and what cannot be synthesized is not a sharp one. Marked species variations exist and even within the same species a variety of circumstances determine whether a particular factor can be synthesized in amounts which are adequate, inadequate, or not at all. It has therefore become customary to designate as a vitamin any factor which may under particular conditions be required as an accessory food constituent. The present discussion includes factors of the B group which clearly satisfy that criterion.

Synthesis of vitamins in the body may occur in the tissues themselves or through the agency of parasitic micro-organisms. In the case of vitamin C, animals which do not require it in their diet, such as the ruminants and rodents, appear to synthesize it in their tissues. With the B group, however, such synthesis as occurs is brought about largely by micro-organisms. A

complete consideration of this field should include not only the accomplishments of the intestinal bacteria, but micro-organisms in general, regardless of their habitat. We shall limit ourselves to such knowledge as is available about the production of B vitamins by intestinal bacteria.

As is the case with most scientific developments, our knowledge of this subject has proceeded in several distinct and logical phases. Early experiments on the exclusion of bacteria in the neonatal period laid the foundations of the subject some time before the existence of accessory food factors was known. The discovery of the vitamins was promptly followed through by workers in plant and animal nutrition who furnished evidence that these growth stimulating substances were produced by bacteria. The identification of the various members of the B complex ushered in a new phase—the study of the synthesis of these specific entities in the gastrointestinal tract of various experimental animals and finally in man. Lastly, the discovery of intestinal bacteriostatic agents—notably the insoluble sulfonamides—has provided the investigator with a new tool for studying this phenomenon. We shall attempt to outline briefly the development of the subject through these different phases.

## II. HISTORICAL BACKGROUND

The belief that the intestinal bacteria exercise an important and perhaps vital function was freely expressed by Pasteur (1) and a number of studies to test this point were undertaken during the latter part of the nineteenth century. Nuttall and Thierfelder (2) observed impaired growth of guinea pigs raised in a sterile environment for 13 days. Schottelius (3) found that sterile chicks died regularly within 17 days, and similar untoward results were noted in frog larvae by Mme. Metchnikoff (4) and in toad larvae by Moro (5). The most convincing studies of this kind were carried out somewhat later by Schottelius (6) who inoculated ailing and poorly growing sterile chicks with pure cultures of colon bacilli with resulting prompt recovery. In these experiments the beneficial effect of the intestinal bacteria might conceivably have been due to direct digestion of food rather than to the elaboration of some essential assimilable factor.

More definite evidence that intestinal bacteria elaborated a nutritive factor was obtained by Osborne and Mendel (7) in 1911. They noted that rats on certain purified diets declined in weight and became coprophagous. The decline in weight could be arrested by incorporating in their diet feces from normal rats. When the rats were offered a choice between their own feces and those of normal rats, they invariably chose the latter. The superiority of the feces from the normal rats was attributed to the bacterial flora which, as was then well known, was greatly influenced by diet (8). Sterilization of the feces reduced their beneficial effect but did not abolish

it, indicating that a thermostabile factor was concerned in addition to either the living organisms or some thermolabile product.

The first indication as to the type of nutritive factor produced by bacteria came, interestingly enough, from a plant physiologist, Bottomley, in 1914 (9, 10). He observed that the addition of bacteria to peat stimulated the growth of wheat seedlings. The growth promoting factor could be concentrated by chemical procedures similar to those used by Funk (11, 12) in purifying his recently discovered "vitamin" (later known as vitamin B)—a rat growth promoting factor. In testing his fractions, Bottomley used seedlings and also a micro-organism, *Azotobacter chroococcum*—probably the first instance of microbiological vitamin assay.

A completely independent study implicating the intestinal bacteria was that of Theiler and his associates (13) in ruminant animals. In the attempt to reproduce experimentally a South African paralytic disease known as "lamzietke" they fed cattle an experimental diet deficient in vitamin B and noted that evidences of deficiency failed to develop although pigeons on the same diet developed polyneuritis promptly. They assumed that either the vitamin requirement of cattle was extremely low or that it had been satisfied by bacterial synthesis in the intestinal tract; the latter explanation seemed the more probable.

Thus far, although bacterial synthesis of vitamins was strongly suspected, there was no evidence that a specific bacterium produced a substance capable of promoting animal growth. The first demonstration of this kind appears to be that of Pacini and Russell (14) who showed that typhoid bacilli contributed to the medium in which they were grown a factor which stimulated the growth of rats. Their reason for choosing this particular organism was the prevalent belief that patients with typhoid fever grew from one to several inches during the course of their illness. Similar results with unidentified bacteria (symbiotes) were shortly afterward reported by Bierry and Portier (15).

### III. COPROPHAGY AND REFECTION

Although Osborne and Mendel had shown that feces from healthy rats contained a growth factor, it remained for Portier and Randoin (16) to demonstrate that feces from animals on a deficient diet also contained the protective factor, thereby eliminating the possibility that the effect was due to any unabsorbed dietary residue or excreted surplus in the animals yielding the feces, and pointing strongly to intestinal synthesis in the deficient donor animals.

In 1923, Steenbock and his coworkers (17) observed that rats on a deficient diet showed improved growth when allowed access to their own feces. The effect could be duplicated by incorporating yeast in the diet



and was therefore attributed to production of the B factor in the intestine. This effect of autocoprophagy was subsequently observed by a number of other investigators (18-22) including Heller, *et al.* (23) who carried the observations further and adduced evidence implicating spore bearing bacteria. It is clear that autocoprophagy is protective against B deficiency, but does not provide complete protection (24-26).

In 1928, Fridericia (27) described what he believed to be a new protection phenomenon which he called '*refection*.' The observation was made that young rats reared on a B-deficient diet containing rice starch failed to thrive except for a few individuals that promptly resumed normal growth and at the same time exhibited characteristic pale bulky feces with a high content of starch. This protective phenomenon could be transmitted from animal to animal by feeding feces. Fridericia showed that the agency of refection did not pass a Berkefeld filter and was thermolabile. He therefore advanced the hypothesis that it was due to implantation of B-producing micro-organisms in the gut.

The phenomenon of refection was studied by a number of other workers (28-33) some of whom were able to reproduce it (28, 30, 34, 35) and others not (36). It occurred only when the diet was high in starch, notably when potato starch was fed. Although Fridericia (27, 37) showed that the reaction and the bacterial flora of the cecum of 'refected' rats differed from controls, he was unable to incriminate particular bacterial species, nor could he show that the intestinal contents of refected animals were richer in vitamin.

It is still not clear why on a particular diet certain animals will become 'refected' and others not. The original hypothesis that the phenomenon is produced by implantation of a B-producing flora provides the most plausible explanation, but is still unproved. The observations on refection were valuable, however, in that they led to other work which threw considerable light on the phenomenon of biosynthesis, notably that of Guerant, Dutcher and their collaborators (25, 26, 38, 39) at Pennsylvania State College. These workers demonstrated that both vitamin B and G were synthesized in the cecum of rats when fed on diets containing dextrinized starch, that some synthesis occurred when the carbohydrate furnished was lactose, but not with other simple sugars. The ceca of animals on the dextrinized starch diet were enlarged and showed a preponderance of yeast on culture in contrast to those of the animals on simple sugars which showed a preponderance of colon bacilli. Cecectomy was found to diminish the protective power of the feces against B deficiency except in instances where part of the remaining colon assumed the structure and function of the cecum (38, 40).

Of equal importance to this work on the rat were the work of Bechdel and his collaborators (41-43) in cattle. Following the early observations

of Theiler (13) mentioned above, they were able to rear calves to maturity on a B-deficient diet, and showed that these animals reproduced normally and after freshening secreted milk only slightly below the normal in B content. They further located the cause of the protection in the bacteria of the rumen.

The past decade, in which such marked progress has been made in separating and identifying the different vitamins of the B group, has brought forth a number of studies on intestinal biosynthesis in animals and, more recently, in man. Many bacteria have been studied in pure culture. The development of accurate assay methods and the discovery of the intestinal bacteriostatic agents used with notable success in this field by Elvehjem and his collaborators (44, 45) as well as Sebrell and his co-workers (46, 47), have been invaluable tools in contributing to the rapid progress that has been made in the past few years.

#### IV. THE BACTERIAL SYNTHESIS OF THIAMIN IN ANIMALS

In 1914, Cooper (48) showed that alcoholic extracts of fowl's and rabbit's excreta possessed antineuritic potency. He postulated that although part of the vitamin content of the stools was derived from the unabsorbed residue, a substantial part was probably derived from bacterial synthesis. However, extracts of *B. coli* failed to exert a measurable antineuritic activity. Guerrant and Dutcher (24, 25) later were able to show that the stools of rats on a B-deficient diet contained appreciable thiamin. The vitamin was apparently utilized by the screened animal, inasmuch as its requirement for the vitamin (26, 38) varied inversely with the extent of synthesis. The location of maximum synthesis (39) was found to be the large colon, particularly the cecum. It seemed probable that in cecectomized animals, part of the colon may assume the function of the cecum (38, 40).

Extensive and more detailed studies have been performed on thiamin synthesis in the rumen. McElroy and Goss (49, 50) fed ewes a purified ration for 30 days and found that the amount of thiamin per gram of dry material was over ten times as high in the rumen content as it was in the feed. They found no measurable synthesis in fistulated cows on a thiamin poor ration, although the milk secreted contained an adequate amount of thiamin (50). Wegner, *et al.* (51, 52) were able to demonstrate thiamin synthesis in the rumen of a fistulated calf. Others (53) have observed this phenomenon in most instances, but not in all. Apparently synthesis of thiamin does take place in the gastro-intestinal tract of the cow and can often be demonstrable in the rumen. In this connection a study of this phenomenon in all sections of gastro-intestinal tract of ruminants should be quite informative.

Thiamin synthesis by a wide variety of micro-organisms has been studied,

particularly during the last few years. Among the bacteria that were found capable of producing thiamin are the following: *Pfeiffer's bacillus* (54), *Bacillus vulgatus* (55, 56), *B. proteus*, and *B. subtilis* (57, 58), *B. adhaerens*, (58), *B. lactis aerogenes*, *B. alcaligenes fecalis* (58, 59, 60), some strains of dysentery bacilli (61), strains of diphtherial organisms (62), *B. aerogenes*, *B. vulgatus*, *B. vulgaris*, *B. mesentericus* (60), *E. coli* (58, 60, 63, 64), *Pseudomonas fluorescens*, *Proteus vulgaris*, *Clostridium butylicum* (65), *B. bifidus* (66, 67).

#### V. THE BACTERIAL SYNTHESIS OF RIBOFLAVIN IN ANIMALS

The synthesis of riboflavin, like thiamin, was observed in various animals. In the rat the site of synthesis seems to be the colon (25, 26, 40). When synthesis of riboflavin is increased the requirement of the rat for the vitamin diminishes. In the rumen of sheep (49) there is even greater synthesis. The rumen contents showed a riboflavin value about one hundred times that of the feed used (68, 69). In the cow marked synthesis also takes place (51, 52, 53, 68). The daily output of riboflavin in the milk alone was found to be ten times the intake (68). This impressive amount of synthesis affords adequate explanation for the observation that the riboflavin content of the ration of the cow (70) and goat (71) does not appreciably alter the amount of riboflavin secreted in the milk. Riboflavin synthesis also occurs in the feces of fowl, particularly after passage (72) from the body.

Synthesis of riboflavin by isolated bacteria has also been studied. It was found to be synthesized by the lactic acid bacillus (73), strains of dysentery bacilli (61) and diphtherial organisms (62), *E. coli*, *B. aerogenes*, *A. fecalis*, *B. vulgatus*, *B. mesentericus*, *B. vulgaris* (60), *Pseudomonas fluorescens*, *Proteus vulgaris*, *Clostridium butylicum* (65).

#### VI. THE BACTERIAL SYNTHESIS OF NICOTINIC ACID IN ANIMALS

Numerous workers (72, 74, 75) have reported that the rat can thrive well on a black tongue producing diet. Balance studies (76) showed that rats excreted as much as 40  $\gamma$  of nicotinic acid in excess of the intake. The fecal output alone was three times the intake, a strong evidence for intestinal synthesis which has been shown to occur in the cecum (77). It has also been claimed to take place in the tissue of the rat (78). Ruminants (79) also thrive well for a long period on a ration deficient in nicotinic acid with no ill effects. In sheep the excretion of nicotinic acid (80, 81) remains substantial despite a deficient intake and the nicotinic acid level in the blood (81) is comparable in value to that of animals fed a stock ration. A study of the rumen of the calf (51, 52) showed clearly that nicotinic acid is synthesized to a considerable extent. Similar results were obtained in the cow (76).

Although there is little doubt that nicotinic acid synthesis takes place in the rat, sheep, cow, and other animals, the simple measurement of nicotinic acid excretion does not seem to be an adequate index. From all available evidence the only index of nicotinic acid deficiency appears to be the urinary  $F_2$  fraction of Najjar and Wood (82-86). This was confirmed by workers using the  $F_2$  assay (87, 88) and the so-called trigonelline fraction (89, 90) which is mainly, if not all,  $F_2$  (91, 92). The  $F_2$  fraction, a derivative of  $N^1$ -methylnicotinamide (86, 91, 93-95) was found to be excreted in substantial quantities in rats and pigs while on a nicotinic-acid-deficient diet, a fact which indicates synthesis of the vitamin.

Numerous bacteria have been found to synthesize nicotinic acid in pure media. *E. coli*, *B. aerogenes*, *A. fecalis*, *B. vulgatus*, *B. mesentericus* (60), *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Proteus vulgaris*, and *Clostridium butylicum* (65).

#### VII. THE BACTERIAL SYNTHESIS OF PYRIDOXIN IN ANIMALS

Most of the work on the synthesis of pyridoxin has been performed in ruminants, although it has been shown to occur in the cecum of the rat (77). There is a considerable increase of this vitamin in the rumen and reticulum content of ewes as compared to the feed (49). Similar synthesis has been observed in the rumen of the calf and the cow (68, 51) to an extent that milk from a cow (68) on a deficient ration contains about as much pyridoxin as that secreted by cows consuming a normal ration.

The common bacteria that have been shown to synthesize this vitamin are: *Pseudomas fluorescens*, *Proteus vulgaris* and *Clostridium butylicum* (65).

#### VIII. THE BACTERIAL SYNTHESIS OF PANTOTHENIC ACID IN ANIMALS

Shortly after the identification of pantothenic acid with the chick antidermatitis factor (96) and as a necessary vitamin for the rat (97), evidence for its synthesis was reported in the rumen of ewes (49), cows (68), and calves (51). This synthesis is evidently appreciable since the pantothenic acid output in milk alone was about twice the intake (68). *Pseudomonas fluorescens*, *Proteus vulgaris*, and *Clostridium butylicum* were found to synthesize pantothenic acid in a pure medium (65).

#### IX. THE BACTERIAL SYNTHESIS OF BIOTIN IN ANIMALS

In the rumen of the calf (51) as well as that of the cow (98) biotin was found to be synthesized in considerable amounts. The rumen contents sampled four hours after feeding showed about five times the value of biotin as compared to that of the feed (51).

Rats have been shown to thrive well on a biotin-free diet. However, when sulfaguanidine or succinylsulfathiazole is incorporated in such a diet (44-46) a deficiency develops which can be prevented or cured by the

administration of biotin. Furthermore, rats on a biotin-low ration excrete the vitamin in amounts greatly in excess of the intake (45). Such synthesis was depressed after the administration of sulfaguanidine, but not totally abolished. The cecum of the rat is again the main location for synthesis. The bacteria (60, 65) that have been shown to synthesize biotin in pure media are: *E. coli*, *B. aerogenes*, *A. fecalis*, *B. vulgatus*, *P. vulgaris*, *Pseudomonas fluorescens*, and *Clostridium butylicum*.

#### X. THE BACTERIAL SYNTHESIS OF FOLIC ACID IN ANIMALS

The rat apparently can synthesize folic acid to supply its requirements of this vitamin. No signs of deficiency develop on a ration deficient in folic acid (44, 47, 99, 100). However, when the insoluble sulfonamides are included in the diet of the rat, deficiency develops with poor growth (44), leukopenia, granulopenia (47, 101-103), and achromatrichia (100). All these manifestations of deficiency were corrected by the administration of either crystalline folic acid or folic acid concentrate. The blood picture of dyscrasia was previously observed in the monkey (104-108) and the chick (109) on a diet deficient in the vitamin without necessitating the use of sulfonamide. The deficiency in these animals seems to be a folic acid deficiency (108-110). *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Proteus vulgaris*, and *Clostridium butylicum* were found capable of synthesizing folic acid in a completely vitamin-free medium (65).

#### XI. THE BACTERIAL SYNTHESIS OF INOSITOL IN ANIMALS

The synthesis of inositol by the bacterial flora of the mouse has been definitely established by Woolley (111). Cultures of the intestinal contents of the mouse in a synthetic medium, showed that the intestinal organisms were capable of synthesizing the vitamin. This phenomenon furnished adequate explanation to the observation (112) that deficient mice with alopecia exhibited spontaneous cures while subsisting on an inositol-free regime (113). The amount of the vitamin stored was found to be lower in the bodies of mice with alopecia as compared with that stored by mice showing spontaneous cures. Moreover, the bacterial flora of the former showed poor synthesis as compared with the latter, when such flora was grown in synthetic media.

The bacteria that have been shown to synthesize inositol in a vitamin-free medium are *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Proteus vulgaris*, *Clostridium butylicum* (65).

#### XII. FACTORS INFLUENCING BACTERIAL SYNTHESIS IN THE ANIMAL ORGANISM

The definite influence of the dietary intake on the intestinal flora of widely separated species was clearly demonstrated by Herter and Kendall

(8) using the monkey and the cat as experimental animals. They showed that when both animals were put on a diet high in carbohydrate, the intestinal flora was predominantly an acidophilic non-proteolyzing type. However, on a diet high in protein there was a change to a flora of strongly proteolyzing type. Rettger and Horton (114) found appreciable differences in the intestinal flora of rats receiving an ordinary diet and those fed the experimental diet of Osborne and Mendel (7). On the experimental diet the flora became more simplified with fewer types of bacteria present, and a definite increase of the Gram-positive organisms with a marked increase in *B. bifidus* and a suppression of *B. coli*. There seems to be little doubt that different diets will produce marked differences in the intestinal flora in different species of animals. With this radical change in the flora, one can readily expect a corresponding change in the metabolites and products of those bacteria, vitamins included.

### *1. Carbohydrates, Proteins, and Fats as Influencing Factors*

The influence of isolated carbohydrates in the differential growth of micro-organisms, particularly the enteric group, is well known. Guerrant and Dutcher (25) showed that the ingestion of dextrinized corn starch as the sole carbohydrate in the diet favorably influenced the bacterial synthesis of thiamin and riboflavin in the intestinal tract of the rat, to such an extent as to lower their requirements (26) for these two factors. This enhancing effect of dextrinized corn starch was in sharp contrast to the poor synthesis obtained when commercial starch (undextrinized), sucrose, glucose, and lactose were used. In this connection the results obtained by Najjar and Holt on humans are of interest. One experimental subject who had been for months on a highly purified diet with dextrimaltose (rich in dextrin) as the carbohydrate in the diet, synthesized and excreted in the stools (Table IV) an average of 250  $\gamma$  of thiamin per day. When rice was ingested in place of dextrimaltose for 7 days the amount synthesized dropped to 36  $\gamma$  per day. When dextrimaltose was reinstituted the value went up to 507  $\gamma$  per day. A similar suppression was observed when sucrose was used; however dextrose showed an apparent stimulation. Apparently dextrimaltose and dextrose were more potent in furthering thiamin synthesis. Considering the refection phenomenon as indicative of increased vitamin B synthesis in the intestinal tract of the rat, we observe that such synthesis occurs only when either rice starch (25, 30, 31), dextrin containing starch (27), or potato starch (28, 29) is used. Furthermore, guinea pigs (103) thrive well when 15% of yeast is added to a synthetic diet containing dextrin. However, if dextrin is replaced by sucrose or if sulfaguanidine is added to the diet, they fail to do so. Apparently dextrin promotes bacterial synthesis of an unknown factor not furnished by yeast, which sulfaguanidine suppresses and sucrose fails to promote. It thus

seems reasonably clear from the evidences cited that dextrin and dextrin containing carbohydrates appear to create more favorable conditions for the promotion of synthesis.

The synthesis of riboflavin in the rumen is apparently suppressed (52) by increasing the nitrogen content of the feed. The fat of the diet has been reported (115) to influence thiamin synthesis in the intestinal tract of the rat. The stools of rats on a thiamin-deficient diet containing lard with sucrose as the carbohydrate were potent. When lard was withheld the stools lost their potency. Even roughage (26) has been thought to influence synthesis in the rat by affording more favorable conditions for the growth of micro-organisms. It seems more likely that the inclusion of roughage in the diet, through its laxative effect, results in the presence of more unabsorbed food substances in the colon and therefore a richer substrate for the growth of micro-organisms and increase in synthesis.

## 2. Vitamins As Influencing Factors

The results of Heller, *et al.* (23) suggest that B factors in rat feces were probably concerned in stimulating the growth of spore-bearing organisms which, in turn, were shown to be capable of synthesizing vitamin B. More definite and clear-cut evidence, however, was furnished by Wegner, *et al.* (51). They reported that the addition of thiamin to the feed of a calf apparently stimulated greater production of riboflavin, pantothenic acid, pyridoxin, and biotin. Nielsen and Elvehjem (44) showed that biotin synthesis in the rat was high on a stock diet and low on a pantothenic-acid- or a riboflavin-low ration. Woolley (111) obtained greater synthesis of inositol when pantothenic acid was added to the diet of experimental mice. Furthermore, inositol-deficient hairless mice receiving pantothenic acid but no inositol (112), in many instances regained their hair, suggesting that inositol synthesizing micro-organisms require pantothenic acid for effective growth and possibly riboflavin as well. However, there is room for speculation as to why mice develop alopecia when given inositol but not pantothenic acid unless both are intimately involved in hair growth or pantothenic acid is necessary for the absorption of inositol (116). Welch and Wright (117) showed that the vitamin K deficiency in rats induced by sulfasuxidine can be corrected by supplementing the diet with folic acid and biotin without resorting to vitamin K administration. The effect thus exercised is presumably exerted through stimulation of bacterial synthesis of vitamin K by organisms not too sensitive to the action of the sulfonamide (118). This probably holds true unless the folic acid and biotin are involved in the utilization of vitamin K, a possibility that does not seem likely.

Martin (119) showed that under the conditions of his experiment rats

grow well on a diet containing thiamin, riboflavin, nicotinic acid, pantothenic acid, pyridoxin, and choline. The addition of both inositol and *p*-aminobenzoic acid did not produce a more favorable growth. However, the addition of either inositol or *p*-aminobenzoic acid alone precipitated a need for the other. This was thought to be due to alteration of intestinal synthesis produced by the administration of one vitamin or the other.

That one organism needs a certain particular growth factor in order to synthesize another (120) is clearly exemplified in synthetic media. Kögl and Fries (121) showed that two different fungi can grow well together in a synthetic medium because one requires thiamin and the other needs biotin and the required vitamin in each case is furnished by the other fungus. Neither of the two fungi could grow in a synthetic medium in pure culture. Even more striking is the work of Müller and Schopfer (122), who showed that a yeast and a mold in a synthetic medium grew well together in symbiosis, because one supplied a component of thiamin which the other needed but was not able to synthesize. This, at least, should furnish one explanation for the symbiotic phenomena so often observed between micro-organisms (123), micro-organisms and plants (124), as well as micro-organisms and animals as we have attempted to show in this review.

### 3. *The Role of Sulfonamides as Influencing Factors*

With the introduction of sulfanilylguanidine (sulfaguanidine) by Marshall and his associates (125) as a poorly absorbed anti-bacterial agent, it became possible to study not only the separate vitamin requirements in animals but also the possible extent of synthesis that might take place. By merely suppressing such synthesis, obvious signs of the particular deficiency develop. It also affords opportunities for the study of the requirements of a vitamin on different dietary regimes by abolishing, whenever possible, the interfering factor of synthesis. The metabolic interrelationships suspected between the different components of the B complex can also be studied more comprehensively. Most, if not all, of the work suggestive of this phenomenon is doubtless jeopardized by the fact that intestinal synthesis might vary with the conditions used. Thus the development of signs of thiamin deficiency in a pellagrin (126-128) after the administration of nicotinic acid and *vice versa* may not only be due to greater demand for one when the other is administered but to possible suppression of synthesis. There is strongly suggestive evidence, however, that a metabolic relationship does exist between thiamin and nicotinic acid (129, 130), pyridoxin and pantothenic acid (131) and others not within the scope of this review.

The use of the insoluble sulfonamides for the study of intestinal synthesis has been most productive. The demonstration that folic acid and biotin



are synthesized (44, 45, 132) in the intestinal tract of the rat has been made conclusive. The failure in growth following the administration of the drug was promptly corrected by liver extract and only partially by *p*-aminobenzoic acid. When the sulfonamide was withdrawn there was no immediate growth response. All this pointed to the fact that bacterial synthesis in the intestines was responsible for some factors supplied by liver other than *p*-aminobenzoic acid. It was subsequently found that folic acid concentrate and biotin corrected the deficiency produced by either sulfaguanidine or sulfasuxidine. Thus the rôle of folic acid and biotin in the nutrition of the rat was clarified and their synthesis established. Similar deficiencies were reported (46, 78, 133, 134) with blood dyscrasia (47, 135, 136) and vascular changes (137). The effect of the drug was not a toxic one but primarily an inhibition of intestinal synthesis. This was shown (138) by a study on rats fed sulfaguanidine in a synthetic diet with added vitamins except folic acid. There was a positive response in growth upon supplementation with yeast and with feces of rats not ingesting the drug. However, supplementation with stools of sulfaguanidine rats produced no response. Although it is possible that the sulfaguanidine excreted in the feces of the drug-fed rats was partly responsible for the lack of response, there is little doubt that the effect is primarily a suppression of synthesis rather than toxic. The use of liver extracts and *p*-aminobenzoic acid parenterally in such experiments could afford additional conclusive proof of the drug action. Uncovering the rôle and synthesis of biotin and folic acid with the use of the insoluble sulfonamides brought to light a strong suggestion that newer factors, as yet undiscovered, are suppressed by the drugs. It is suggested that there might be more than one new factor in liver or the extract (110, 139) and folic acid concentrate (100) that will counteract the effect of sulfasuxidine. Since *p*-aminobenzoic acid has been shown incapable of counteracting the deficiency produced by sulfasuxidine (133, 140, 141), it becomes quite probable that there might be a multiplicity of factors that liver or yeast supply which the drug suppresses through inhibition of intestinal synthesis.

#### *4. Other Factors Affecting the Vitamin Economy in the Intestines*

The accumulated literature hitherto cited on animals leaves little room for doubt that bacterial synthesis of vitamins in the intestinal tract of different species of animals does take place to a considerable degree. The *quantity* synthesized of a particular vitamin appears to be of considerable importance. In many instances it is enough to supply the need of the animal for that vitamin with an adequate margin of safety. In other instances, the synthesis is totally inadequate to supply the quantity required for normal

well being. In few cases, however, the amount synthesized of a vitamin is so close to that required by the organism, marginal and fluctuating, that in a group of animals under the same conditions some would develop deficiency while others would not. Thus, among a number of chicks (142) on a vitamin-K-deficient diet, some will develop hemorrhagic disease and others will escape; still others will show spontaneous recovery after having exhibited symptoms. An identical process occurs in mice on an inositol-free diet (111, 112). Furthermore the quantity synthesized *per se* does not necessarily explain why some animals require a certain vitamin and others do not. The *location* of synthesis, therefore, seems to be of paramount importance since synthesis might occur in places where most of the vitamins are absorbed or where little or no absorption can take place. It is indeed noteworthy that ruminants are not in the least dependent on their vitamin intake. Bacterial synthesis in the rumen is adequate and so high up in the tract that it becomes available for absorption by the intestinal mucosa. It is furthermore possible that synthesis might very well take place below that level and still be of use to the animal. Non-ruminants, not so favorably endowed, are dependent on the intake of more than one vitamin, although synthesis of others completely furnishes adequate amounts. One significant factor, however, should be well considered, *i.e.*, the difference in the *absorption* of the various vitamins. The fact that the cecum is the main synthetic apparatus of the rat has been definitely established (38) and repeatedly observed (40, 77). In this organ (77) biotin, folic acid, pantothenic acid, and pyridoxin were shown to be synthesized to a greater extent than other vitamins. Deficiency of the first two can be produced only with sulfonamides, whereas the last two can be produced by dietary deprivation. It would seem, then, that most probably biotin and folic acid are absorbed adequately to cover the need of the animal, but pyridoxin and pantothenic acid, though similarly synthesized in substantial quantities, are not as well absorbed since a deprivation of one or the other of these two from the diet will result in deficiency. This explanation holds true provided the requirements of B<sub>6</sub> and pantothenic acid are not well beyond such amounts as are normally synthesized.

### XIII. BACTERIAL SYNTHESIS OF THIAMIN IN MAN

Najjar and Holt (143) have shown in their experimental subjects: (a) that appreciable thiamin was found in the stools of human subjects, (b) that such thiamin did not comprise the unabsorbed residue from the diet, (c) that it was not an excretion product of the body into the stools, (d) but actually synthesized by bacteria, (e) and that it is utilized by the organism to furnish at least part of the required thiamin.

Nine young adults ingested for a period of 18 months a synthetic diet consisting of vitamin-free casein, crisco, and dextrimaltose,<sup>1</sup> including salt mixture and a vitamin mixture, consisting of a constant intake of all the available crystalline vitamins, including decreasing amounts of thiamin. Towards the end of the experiment when the thiamin intake was dropped to the level of 187–230  $\gamma$  daily, which was only the amount supplied by the dextrimaltose, the study of the stool thiamin was started (Table I). All subjects ultimately developed signs of deficiency except one, G. B., who remained for a period of three months on an intake of 187  $\gamma$  daily until

TABLE I  
*The Relation of Free Thiamin Output in Stools to the Onset of Symptoms*

Subject	Thiamin Intake $\gamma$ per day	Free Thiamin Excretion in Stools $\gamma$ per day							
		Periods in Weeks							
		I	II	III	IV	V	VI	VII	VIII
C. G.	230	(9.8)	(0)	(8.5)	Rx	Rx	Rx	Rx	Rx
H. K.	230	(5)	(0)	(4.5)	—	(16)	(2.4)	Rx	Rx
A. P.	187	(4.7)	(1)	(5.5)	Rx	Rx	Rx	Rx	Rx
R. A.	187	(11.5)	(3)	(15.5)	Rx	Rx	Rx	Rx	Rx
S. B.	230	—	38	90	Experiment Discontinued				
C. P.	230	250	—	507	(40)	(69)	Rx	Rx	Rx
G. B.	187	52	133	37	—	—	148	106	146
D. K.	230	143	36	182	(17)	(12)	Rx	Rx	Rx
I. S.	187	53	37	43	(16)	(27)	(6)	(9.6)	(22)

Rx, Therapy instituted.

( ), Presence of symptoms.

—, No collection made.

In each case where symptoms were present the daily thiamin output was below 30  $\gamma$  with the exception of C. P. It is noteworthy that during his symptomless periods, C. P. showed a much higher level of output as compared to the other subjects.

the experiment was terminated, at which time he was vigorous and healthy in every respect.

The amount of free thiamin synthesized in the stools appears to bear a definite relationship to the presence or absence of symptoms. The thiamin output in the stools before the development of symptoms was relatively high,—above 30  $\gamma$  daily. However, with the reduction of thiamin output to a level below this figure, there was a simultaneous development of

<sup>1</sup> The casein obtained from the Sheffield Farms Co., Inc., contained no measurable thiamin, but 0.75–1.0  $\gamma$  per gram of riboflavin. The dextrimaltose used was found to contain no measurable riboflavin but 0.6–0.9  $\gamma$  per gram of thiamin

symptoms. This finding was consistent in all subjects during the periods of observation, with the exception of C. P. It is well to point out that this subject showed a much higher level of output of free thiamin than the others, before as well as after the development of symptoms. The fall in his thiamin output was, however, comparable in magnitude to that of the others. It is quite significant that G. B., the only subject who maintained a consistently high level of excretion throughout the experiment, did not show any signs of deficiency while subsisting for three months on an identical diet.

That bacteria were responsible for synthesis of thiamin in the intestinal tract was proved through the use of sulfasuxidine as a bacteriostatic agent. Two subjects (Fig. 1) were given 1.5 g. of the drug every four hours. A zero level of excretion was reached on the fourth and sixth day. Twelve

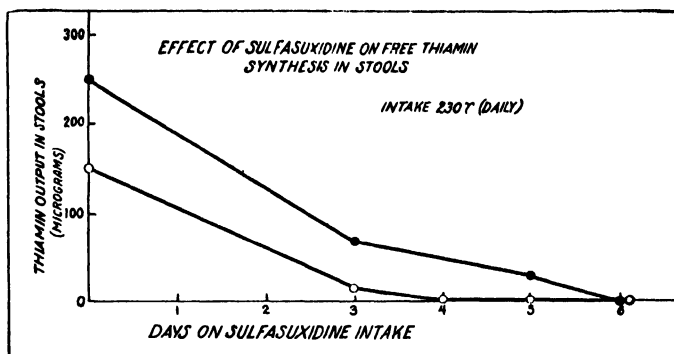


FIG. 1

other subjects receiving the same diet with daily thiamin intake of 1230  $\gamma$  also showed zero excretion of free thiamin after sulfasuxidine therapy. The output of combined thiamin took a longer time to reach minimal level, although greatly reduced, it did not reach zero level in six days.

The possible destructive effect of the drug on thiamin *per se* was ruled out. Further proof of synthesis was the fact that in seven subjects out of nine there appeared, in many instances, more total thiamin (free and combined) output in the stools alone than there was in the food eaten. In almost every instance, however, the total urine and stool output of total thiamin exceeded the intake. The possibility that thiamin in the stools represents an *excretion product* from the body into the gut was ruled out. As is clearly shown in Table II there was no increase in stool thiamin after intravenous administration of the vitamin. The other possibility that the stool thiamin might comprise the unabsorbed residue was explored and

excluded. The total thiamin output was, in many instances, higher than the intake. Following administration of sulfonamide, the thiamin stool output was abolished, indicating that none of the ingested thiamin reached the lower gut even when the intake of thiamin was above one milligram. All the ingested thiamin was absorbed. The thiamin so synthesized in the bowel is apparently absorbed and utilized by the organism. The correlation between the presence of symptoms of deficiency, along with a low stool thiamin and the lack of such symptoms with a high thiamin output, is indeed highly suggestive. Furthermore, the development of symptoms simultaneous with the drop of stool output to a deficiency level lends more emphasis to this point, particularly when it is realized that the subjects

TABLE II  
*Thiamin Output in Stools Following I.V. Injection of 50 mg. of Thiamin*  
Micrograms per day (Weekly Specimens)

Subject	Before Injection	After Injection
H. K.	4.5	2.7

TABLE III  
*Urinary Excretion of Thiamin Following Thiamin and Cocarboxylase Enemata*  
(Micrograms in 12 hrs. Specimen)

Subject	Enema	Urinary Output	
		Before Enema	After 2nd Enema
A. P.	Thiamin 50 mg.	160	1,615
S. B.	Thiamin 50 mg.	162	5,200
C. P.	Cocarboxylase 50 mg.	55	44

were at about the critical level of intake, and for a long period were ingesting a minimum amount. More concrete evidence of utilization of the synthesized thiamin in the gut was produced by the use of high enemata. It is clear from Table III that good absorption of free thiamin took place through the gut but no absorption of combined thiamin (cocarboxylase) occurred. This explains the good correlation that existed between symptoms and free thiamin output and absence of such correlation with combined thiamin. A study of the stool thiamin output from a low ileostomy fistula in a baby showed the presence of measurable thiamin which was abolished by the use of sulfasuxidine. Apparently thiamin synthesis is not confined to the large gut, but occurs also in the ileum where good absorption would be expected.

An attempt was made to study the influence of different carbohydrates

on the synthesis of thiamin. Four subjects that had been ingesting dextrimaltose as the sole carbohydrate in the diet were given different carbohydrates as a substitute (Table IV). It seems that rice and sucrose produced a diminution of the amount synthesized, while dextrose produced an apparent stimulation. The reduction produced by rice appears to be significant and indeed challenging, inasmuch as beriberi has long been known to be prevalent among rice consuming communities. It is common knowledge, of course, that rice *per se* is almost devoid of thiamin.

TABLE IV

*The Effect of Different Carbohydrates Ingested for One Week on Thiamin Synthesis in the Stools*

Free Thiamin Output in Stools  
(Micrograms per day)

Subject	Dextrimaltose	Carbohydrate Substituted		Dextrimaltose
C. P.	250	Rice	30	507
D. K.	143	Sucrose	35	182
J. S.	53	Maltose	37	43
G. B.	52	Dextrose	133	37

#### XIV. BACTERIAL SYNTHESIS OF RIBOFLAVIN IN MAN

The synthesis of riboflavin was investigated (144) using a procedure similar to that employed for thiamin. A synthetic diet supplemented with crystalline vitamins was ingested for a period of twelve weeks and furnished 70-90  $\gamma$  of riboflavin daily. Riboflavin was found to be synthesized in much greater amounts than thiamin. The daily urinary output alone was two to three times the intake and the combined excreta averaged five to six times the amount ingested. The daily fasting hour excretion (Fig. 2), after a preliminary drop, was maintained throughout at a level well within the normal range. When the subjects were given sulfasuxidine there was no suppression of synthesis, notwithstanding its continuous intake for a period of five weeks. In fact, the only effect, if any, was an increase in the riboflavin output in the stools, although synthesis of free thiamin in the same subjects was promptly abolished. The riboflavin in the stools was shown not to be an excretion product of the body into the bowel. With this evidence at hand it seems likely that this factor was synthesized mainly by organisms not susceptible to the drug. Indeed smears and cultures of the stools revealed the presence of an abundance of bacteria, a clear indication that the drug did not eliminate all bacterial growth and accounts for the persistence of riboflavin synthesis.

Bacterial studies have shown *B. coli communior* to be predominant in

the subjects that have thus far been studied. It is clear (Fig. 3) that *B. coli communior* is capable of synthesizing appreciable amounts of riboflavin. It appears that as the organism multiplies to reach a peak count after

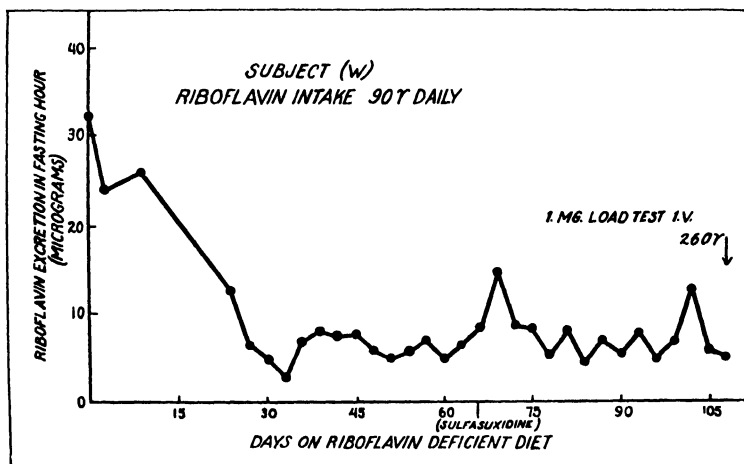


FIG. 2

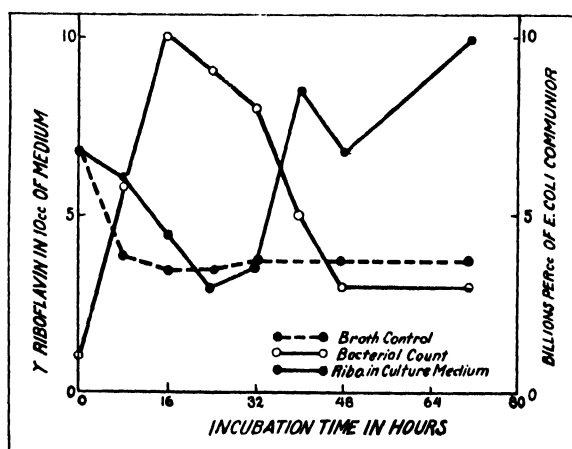


FIG. 3

16-24 hours incubation, the riboflavin content of the medium shows a corresponding and gradual decline. With continued incubation the count declines steadily and the riboflavin content shows a corresponding increase. The uninoculated broth control showed a drop during the first eight hours

of incubation to a level that was maintained throughout. This behavior suggests that most, if not all, of the riboflavin is synthesized and held within the bodies of the bacteria. Liberation into the medium occurs after bacterial disintegration.

#### XV. BACTERIAL SYNTHESIS OF NICOTINIC ACID IN MAN

Ellinger and Coulson (145) in a recent report showed that the urinary output of *N*<sup>1</sup>-methylnicotinamide chloride, an F<sub>2</sub> compound (86, 146, 147), was markedly diminished when sulfasuxidine or sulfaguanidine were administered to subjects subsisting on a uniform diet and environment throughout the experiment. The reduction in output was not the result of an interference of the drug with nicotinamide metabolism. Control subjects, who were given the soluble sulfathiazole, showed higher drug levels in the blood and failed to show a reduction in the output of *N*<sup>1</sup>-methylnicotinamide chloride. The authors concluded that suppression of synthesis of nicotinamide in the intestinal tract produced by the insoluble sulfonamide was an obvious explanation of the results obtained. The results are undoubtedly very suggestive of synthesis, although interference of absorption of the vitamin cannot be excluded, because the diet used was apparently not vitamin-free.

Najjar and Holt (155) studied the urinary excretion of the F<sub>2</sub> compound on four subjects who ingested a diet of Crisco, Vitamin-free Casein (Sheffield) and Dextrimaltose no. 2 for a period of three months. The daily urinary output of F<sub>2</sub> was equivalent to the excretion response after the intake of 20–30 mg. nicotinamide, although the daily intake of each subject amounted only to 1.5–2.0 mg. derived from the Dextrimaltose<sup>2</sup> and the casein.<sup>3</sup> When full doses of sulfasuxidine were given for a period of a month, none of the subjects showed a decrease in the F<sub>2</sub> output. These results are indicative of synthesis of the vitamin although sulfasuxidine failed to inhibit the process.

#### XVI. BACTERIAL SYNTHESIS OF BIOTIN AND FOLIC ACID IN MAN

Oppel (148) produced quantitative evidence in respect to biotin. Human subjects were found to excrete in the urine more biotin than they ingested in the diet. The stool output of biotin also was more than the amount ingested. Both urine and stools contained three to six times the biotin content of the diet. The conclusion was therefore drawn that bacterial synthesis was responsible. The results of Najjar and Holt (143) contain

<sup>2</sup> The nicotinic acid assay on Dextrimaltose no. 2 was obtained through the courtesy of Dr. Warren M. Cox, Jr., Mead Johnson and Company. Each gram of Dextrimaltose contained 8.6 microgram.

<sup>3</sup> The nicotinic acid assay on the casein was obtained from the Sheffield Farms Co., and reported to contain 0.3–0.9 gamma per gram.



strongly suggestive evidence that the bacterial synthesis is quite adequate to take care of the need of both biotin and folic acid by man. Their subjects subsisted on a synthetic diet which did not include biotin and folic acid in the vitamin supplements. The diet, except for dextrimaltose, was not materially different from that used to produce biotin and folic acid deficiencies in the rat (47, 132) when synthesis of these two vitamins was abolished with sulfonamides. The daily intake of folic acid furnished by dextrimaltose<sup>4</sup> amounted only to 14–17  $\gamma$  for each subject. The other two items of the diet, Vitamin-free Casein and Crisco, might have contributed traces of the vitamin if at all. These subjects were kept on the diet for eighteen months, during which time there was no evidence of any blood changes, skin lesions, electrocardiographic or X-ray changes, nor any arterial damage that could be detected or affect the blood pressure, as repeated measurements of blood pressure were normal.

#### XVII. VITAMIN SYNTHESIS AND ITS IMPLICATIONS IN HUMAN NUTRITION

The ability of humans to synthesize thiamin, riboflavin, nicotinic acid, and folic acid through bacterial action has been discussed. This fact in itself presents a new approach and a different outlook on the controversial subject of "Vitamin Requirements in Man." It affords, to begin with, an adequate explanation for the different results obtained by different workers (149–152). From the data heretofore shown on thiamin (143) and riboflavin (144) one is impressed by the fact that even under identical conditions of diet and environment there was a good deal of variation in the quantities synthesized by one individual as compared with another. One subject would show consistently high values while another consistently low ones. Since such vitamins are absorbed and therefore used to advantage, one could certainly expect a smaller dietary need for one subject and a higher one for the other, assuming that their metabolic requirement for the vitamin in question is similar. We are mindful that this difference in synthesis is but one factor out of a number that might determine the amount required; nevertheless, it is an important one. It acquires even more import when fuller knowledge of the effect of diet on synthesis is obtained. The requirement of a particular factor might be quite different when a high protein, carbohydrate, or fat diet is ingested, not only because of a different metabolic need for the vitamin, but because it might affect synthesis one way or the other. Indeed a favorable dietary regime might markedly lower an otherwise high requirement. In the case of riboflavin (144) the subjects were kept for over three months on an insignificant

<sup>4</sup> Folic acid assay on dextrimaltose (no. 2) was made through the courtesy of Dr. Warren M. Cox, Jr., Mead Johnson and Company. Using *S. lactis* R. the folic acid content was found to be 0.056  $\gamma$  per gram.

intake of riboflavin without showing any clinical or chemical signs of deficiency. The urinary fasting excretion was maintained at a level within the normal range for all this period with no sign of decline (Fig. 2). It is conceivable that had those subjects been allowed to continue on the diet for a year or more, signs of deficiency might develop. It still remains hard to explain the lack of any chemical signs of even reduced stores after as long an interval as twelve weeks. To speculate further, bacterial synthesis might, after intimate knowledge of its workings, be harnessed to supply a larger share of the vitamin required.

Another aspect that this problem presents is a redefinition of what we now call requirement. By nutritional requirements we often infer the amount of a factor that is necessary to ingest in order to maintain a normal state of health. This mode of expression in the light of our present knowledge of bacterial synthesis falls short of expressing the physiological need of the particular vitamin. In cases where no synthesis occurs (and these probably are non-existent) the physiological need of a factor can well coincide with its *nutritional requirement*. The nutritional dietary requirement of biotin is nil, but its *physiological requirement* is qualitatively undebatable and quantitatively not as yet determined. The nutritional requirement of thiamin must be measurably less than the physiological requirement. The difference is accounted for by the amount synthesized. If synthesis is suppressed by the sulfonamides, one can obtain a clear and quantitative estimation of the physiological requirement of thiamin. Thus a clearer understanding of what is implied by *requirements* has become quite necessary, let alone *allowances*, since the exploration of actual *physiological requirements*, at least in animals, lends itself to ordinary experimental procedures.

The widespread use of the sulfonamides and its effect on known and as yet unknown factors is indeed challenging. There is little doubt that the soluble sulfonamides (153, 154) are retained in the intestinal tract in such concentration as to exercise active bacteriostasis. The length of time that such drugs are used ordinarily by a patient can hardly be expected to precipitate a state of deficiency in the average case. As prophylactic agents to a number of diseases, sulfonamides are and will be used for varying lengths of time. The possibility of inhibiting synthesis for periods long enough to have a deleterious effect should certainly not be ignored.

### XVIII. CONCLUSION

The synthesis of B vitamins by bacteria has been reviewed from its early phases. The accumulated knowledge gained through successive research in this field leaves no doubt regarding the fundamental importance of this phenomenon and its vital rôle in the nutrition of plants and animals, in-

cluding man. It is also apparent that those factors synthesized by micro-organisms are not mere by-products of their metabolic activity, but are necessary for their growth and multiplication. In this respect, the metabolic requirements of unicellular organisms for the water-soluble vitamins is quite similar to that of man and other higher forms of life. It is indeed fortunate that bacteria residing in the gastro-intestinal tract do not compete with the animal for these factors but synthesize them in sufficient amounts to render them available to their host. The extent to which this bacterial synthesis is used to advantage by the host seems to depend on the location and extent of synthesis. Ruminants do not seem to be dependent on a dietary intake of these factors because synthesis occurs in the proximal portion of the gut and affords a chance for adequate absorption. Man and the rat are not endowed with a rumen, but have to depend on synthesis in more distal portions of the intestinal tract, mainly the large gut. It is not surprising then to find that, unlike ruminants, they are dependent on their dietary intake for some of the needed vitamins, without which deficiency develops. In such instances, where a dietary supply is necessary, there is little doubt that part of the requirement is, nevertheless, supplied by bacterial synthesis. Other B vitamins, however, are provided adequately without the need of an outside source.

A number of factors have been shown to influence the extent of this bacterial synthesis. The constituents of the diet: proteins, fats, carbohydrates, and the different vitamins appear to play a significant part. The widespread use of bacteriostatic agents (the sulfonamides), particularly in protracted therapy or prolonged prophylaxis, should obviously call for vigilance because of their action in suppressing synthesis of the various members of the B complex.

The phenomenon of vitamin synthesis by bacteria presents important considerations in the nutrition of man. Its full significance, however, should await a more complete knowledge of the human need for the B vitamins than we now possess. At the present time, we are certain that at least part of our requirements are furnished by bacterial action in the intestines. It is, therefore, comforting to know that we can at least rely to some extent on this mechanism to supplement the average American diet should the ever persistent vitamin vendor fail in his benevolent task.

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# Sulfonamides and Vitamin Deficiencies

By FLOYD S. DAFT

AND

W. H. SEBRELL

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## I. INTRODUCTION

In recent years, there has been a growing realization that the sciences of nutrition and of pharmacology have a common frontier. For many decades, we busied ourselves with studies of the dietary needs of the "normal" individual, and with determinations of the toxic or pharmacological effects of drugs on animals receiving an "adequate" diet. Many recent studies, however, have involved the use of drugs in highly purified diets of known composition. Some have been undertaken to determine the effects of these drugs on the nutritive state of animals, others to test the influence of specific nutrients on drug action. In either case, the results of these studies have enriched both sciences and appear to be playing a part in shaping the future of each.

The most widely and successfully used drugs in nutrition experiments are the sulfonamides. Although they have been employed in this way only during the last three or four years, several scores of reports dealing with the results of their administration to rats and other experimental animals have already appeared. A large majority of workers have employed sulfaguandinine or succinyl sulfathiazole (sulfasuxidine), although experiments with sulfanilamide, sulfadiazine, sulfathiazole, sulfapyrazine, and sulfapyridine have been reported.\*

\* See S. Ansbacher, *Vitamins and Hormones* **2**, 215 (1944), and B. C. J. G. Knight, this volume, p. 105.



The historical basis for this use of the sulfonamides is to be found in experiments dealing with the synthesis of vitamins by intestinal bacteria. For such a process to be of nutritional importance to rats or other animals, it is obvious that not only must needed vitamins be manufactured in this way but also they must be made available in significant amounts to the animals in question. It has been shown that both of these conditions are at times fulfilled. The first step was the demonstration, made some 30 years ago, that rats were benefited, under suitable experimental conditions, by the ingestion of feces. It thereafter was indicated by an orderly progression of researches that the benefits experienced were derived from vitamins in the feces, that these substances had been synthesized by intestinal bacteria, and finally that coprophagy was not essential in order for the animal harboring the micro-organisms to profit from their activity.

Osborne and Mendel in 1911 (1) reported that rats receiving special diets and showing "symptoms of nutritive decline" improved clinically and resumed growth following the ingestion of feces from rats on stock diets. They suggested that these effects might be dependent on the living bacteria in the feces ingested. Steenbock, Sell, and Nelson in 1923 (2) found that the growth rate of rats on diets low in "vitamin B" was improved by allowing them to ingest their own feces. They presented evidence to indicate that it was the vitamin content of the feces which was important and suggested that either the vitamin of the food had not been assimilated and was therefore excreted with the feces or that it had served its purpose without being destroyed and was subsequently voided in the urine and absorbed by the feces. Heller, McElroy, and Garlock (3) confirmed and extended the experimental findings of Steenbock, *et al.* In seeking the correct explanation for the presence of "vitamin B" in feces, they considered the further possibility, previously suggested by Cooper (4), that the vitamin might have been synthesized by intestinal bacteria. They were able to obtain evidence, from *in vivo* and *in vitro* studies, which supported the point of view that this mechanism was operative and they concluded that certain unidentified spore-bearing organisms manufactured "vitamin B" in the intestinal tract and stored it within their own bodies.

In 1926 there was introduced into the biochemical literature a concept which was destined to exert a profound influence on our thought concerning the rôle of intestinal bacteria in the nutrition of higher animals. The idea presented was that vitamins might not only be synthesized by the bacteria of the gastro-intestinal tract but might also be released in significant amounts into the surrounding medium from which they could be absorbed and utilized by the host. This mechanism was at first suggested as applying only to rats after the development of a condition designated as "refection." It was later shown to have a more general significance.

Fridericia (5) who made the original observations on the phenomenon of

refection defined it as a transmissible change in the intestinal content, enabling rats to grow and thrive without "B-vitamin" in the food. Fridericia and coworkers (6) demonstrated that while only a few of the rats receiving a deficient diet containing rice-starch became relected spontaneously, the condition could be transmitted routinely to animals ingesting this diet by feeding them feces from relected animals. Such feces were white and bulky and contained a large amount of undigested starch. Fridericia, *et al.* showed further that the relected state could be initiated and maintained only by the use of diets containing uncooked starch. They reported that "vitamin B" was present in the feces of relected rats in comparatively large amounts and that relected animals had an unusual intestinal flora. They suggested that the nature of the intestinal flora might be a critical factor but their attempts to identify the infectious agent of refection were not successful.

Roscoe (7) corroborated many of the findings of Fridericia and coworkers and presented experimental data which suggested that in order for relected rats to continue in that state it was necessary for them to ingest some of their own (or similar) feces. Roscoe also referred to the convincing experimental evidence presented by Goldberger and his colleagues (8) that so-called "vitamin B" consisted of at least two factors, and discussed experiments which demonstrated that relected animals were protected from dietary deficiencies of "both" factors.

In most later experiments refection was prevented by the substitution of other forms of carbohydrate for raw starch. It was found, nevertheless, that even without refection the host animals, both ruminants and to a lesser but important extent non-ruminants, might benefit from the synthesis of vitamins by the bacteria of the digestive tract.

Bechdel, Honeywell, Dutcher, and Knutsen in 1928 (9), searching for an explanation as to why cattle, unlike any other species of animal studied, were able to grow to maturity, give birth to normal offspring and produce milk of normal dietary composition on a ration highly deficient in vitamin B complex, demonstrated, first, that rumen extract from a heifer on a deficient diet contained "vitamin B" and, second, that an organism of the genus *Flavobacterium*, found to be about 90 per cent predominant in the rumen microflora, was able *in vitro* to synthesize "vitamin B". After the demonstration that "vitamin B" is in reality a group of water-soluble vitamins of great complexity, more detailed studies of the synthesis of individual members of the group were undertaken by McElroy and coworkers (10-14), Wegner, Booth, Elvehjem, and Hart (15), and others. It has been shown that biotin (10), riboflavin (and vitamin K) (11), pyridoxin (12), thiamine (13), pantothenic acid (14) and nicotinic acid (15) are all synthesized in the rumen of sheep or cows in amounts sufficiently large to permit these animals to dispense with dietary sources of these vitamins.

Guerrant, Dutcher, and Tomey in 1935 (16) after a consideration of per-

tinent experimental data, stated: "The cow is not unique in its ability to synthesize the B vitamins in its digestive tract. The cow does have the advantage over the rat in that the vitamins are synthesized higher up in the alimentary canal of the cow (rumen), thus enabling this animal to secure (on a ration deficient in the B vitamins) full benefit of the synthesized products without the necessity of reingestion of feces." The difference between the ruminants and non-ruminants is very real. The intestinal bacteria of rats and mice elaborate many vitamins but it has never been demonstrated that a non-refected rodent, without coprophagy, can obtain from this source enough or even a significant portion of its requirements of such vitamins as thiamine, riboflavin, pantothenic acid, and pyridoxin. In the case of both fat-soluble and water-soluble vitamins which are needed in relatively small amounts, however, the situation may be different. There is evidence that bacterial synthesis may furnish the rat or mouse with vitamin K, inositol and possibly other factors in amounts which may approach or even equal the normal requirements of the animal for these substances.

Greaves (17) described the maintenance of rats on vitamin-K-free diets through 3 generations and reported that only 12 of 77 animals exhibited prothrombin values below 50 per cent of normal. In an attempt to explain this phenomenon, he demonstrated not only that the feces of the animals ingesting the deficient diet contained vitamin K but also, by experiments involving section of the common bile duct or the establishment of bile fistulas, that the resistant animals were dependent on this supply of the vitamin. These data, together with the previous report by Almquist and associates (18, 19) that bacteria normally inhabiting the intestinal tract synthesize vitamin K, left little doubt as to the mechanism whereby experimental animals were protected against the development of signs of vitamin K deficiency. It should perhaps be noted in passing that the hemorrhagic diathesis of the new-born, preventable by the administration of vitamin K either to the mother or less effectively to the new-born infant itself, occurs at a time in the life of the infant when the bacterial flora of the gastro-intestinal tract has not yet become established. It is believed that intestinal synthesis of vitamin K in older children accounts for the short period of danger from the hemorrhagic diathesis (20).

Following his demonstration that inositol is an anti-alopecia factor for the mouse (21) and the observation that there were numerous spontaneous cures (22), Woolley made a more intensive study of the latter phenomenon. He was able to demonstrate (23) that mice, when given adequate pantothenic acid, are able to synthesize inositol and add it to their body stores. From his experimental data it appeared that one site of synthesis was probably the intestinal tract and that the organisms responsible were Gram-negative but not identifiable as *E. coli*. Since inositol deficiency could be

produced in a small percentage of mice fed a deficient diet, Woolley concluded that the inositol synthesized by these organisms was either insufficient to meet all of the requirements of the mouse or else the organisms did not become established in all animals.

Similar, although less extensive, data have been reported for biotin and the *L. casei* factor. Landy and Dicken (24) and Burkholder and McVeigh (25) demonstrated the *in vitro* synthesis of biotin by *E. coli* and other bacteria, and Mitchell and Isbell (26) showed that the output of biotin in the urine and feces of their experimental rats was greater than the dietary intake. Thompson (27) demonstrated the *in vitro* synthesis of the *L. casei* factor by organisms normally inhabiting the gastro-intestinal tract. Kornberg, Daft, and Sebrell (28) reported the occurrence of granulocytopenia in a small percentage of rats fed a purified diet and the correction of this condition by crystalline *L. casei* factor. From analogy with the studies on vitamin K deficiency, they suggested that intestinal synthesis of the *L. casei* factor might account for the infrequency of the appearance of the granulocytopenia.

With this background<sup>1</sup> in mind, it is quite understandable why the announcement in 1940 by Marshall, Bratton, White, and Litchfield (29) that a new drug, sulfaguanidine, had been found to be an effective bacteriostatic agent for intestinal organisms should have been received with great interest. The possibility was immediately envisioned that this drug, when incorporated in diets of highly purified food materials, might prove a valuable tool in the production of vitamin deficiencies in rats and other animals. The success of the investigations thus instigated is unquestioned. It is not equally certain, however, that the mechanism of production of the deficiencies is as simple as was first assumed.

The first reported use of sulfaguanidine in nutrition experiments was by Black, McKibbin, and Elvehjem (30). These investigators found that when this drug, at a level of 0.5 per cent, was fed to rats in a purified diet, a diminution in growth rate resulted. When liver extract or *p*-aminobenzoic acid was administered growth was resumed. The effect of liver extract was immediate but that of *p*-aminobenzoic acid delayed. Black, *et al.* interpreted these results as indicating that the sulfaguanidine had inhibited the bacterial synthesis of unknown growth factors which the liver extract supplied and that the administration of *p*-aminobenzoic acid permitted the gradual restoration of the intestinal flora to normal. Mackenzie, Mackenzie, and McCollum (31) using purified diets containing 1 or 2 per cent of sulfaguanidine, confirmed the experimental findings of Black and coworkers and reported further that the thyroid glands of the animals receiving the drug

<sup>1</sup> Additional discussion of this subject will be found in *Vitamins and Hormones*, this volume, pages 23-48.

became hypertrophied and hyperemic. Daft, Ashburn, Spicer, and Sebrell (32) reported extensive hyaline sclerosis and calcification of blood vessels in rats receiving purified diets containing 1 per cent of sulfaguanidine. Welch (33) announced that sulfasuxidine behaved similarly to sulfaguanidine in reducing the growth of young rats fed purified diets but that its action differed from that of sulfaguanidine in that it could not be antagonized by *p*-aminobenzoic acid.

From this beginning, the list of anatomical and physiological lesions resulting from the use of sulfonamides in purified diets was rapidly extended. Black, Overman, Elvehjem, and Link (34) found a state of hypoprothrombinemia in their young rats receiving sulfaguanidine which could be prevented by vitamin K, by *p*-aminobenzoic acid or by a factor in liver extract. Spicer, Daft, Sebrell, and Ashburn (35) reported the development of leukopenia, granulocytopenia, anemia, and hypocellularity of bone-marrow in rats given sulfaguanidine or sulfasuxidine and the prevention of these dyscrasias by certain liver extracts known to contain the *L. casei* factor ("folic acid"). Daft, Ashburn, and Sebrell (36) added to this list of lesions hyaline necrosis and calcification of voluntary muscles, necrosis of heart muscle, hemorrhage into various organs and subcutaneous tissues, liver damage and dermatitis with alopecia. The dermatitis could be cured with biotin. Ashburn, Daft, Endicott, and Sebrell (37) noted that occasionally the necrosis of heart muscle was so severe as to permit beginning or actual cardiac aneurysm. Martin (38) reported that black rats given sulfaguanidine at a level of 1 or 2 per cent in purified diets showed a marked graying, equal in extent to that seen in rats on diets deficient in pantothenic acid. Administration of concentrates of "folic acid" restored the color of the hair. Wright and Welch (39), using sulfasuxidine, also observed the development of achromotrichia and in addition noted the occurrence of porphyrin-caked whiskers. Both conditions could be prevented by the administration of biotin plus concentrates of "folic acid" but not by excess pantothenic acid. West, Jefferson, and Rivera (40) described hemorrhagic necrosis of the adrenals as well as alopecia and pigment deposition on the fur and whiskers as a result of the administration of sulfapyridine. These signs disappeared following treatment with pantothenic acid. Nielsen and Black (41) found that rats given sulfasuxidine developed marked symmetrical alopecia on the belly and on the hind quarters despite the fact that the vitamin supplement which these animals received contained among other factors biotin, pantothenic acid and a superfiltrol eluate ("folic acid" concentrate). Inositol supplementation prevented the onset of this condition. Kornberg, Daft, and Sebrell (42) reported that the use of sulfapyrazine, sulfadiazine, or sulfathiazole resulted in the development of a hypoprothrombinemia so severe that there was a marked prolongation of the clotting time of whole blood as well

as multiple, massive hemorrhages. Kornberg, Endicott, Daft, and Sebrell (93), by lowering the casein content of diets containing sulfadiazine or sulfathiazole, permitted the development of marked lesions of the kidneys. These lesions could be prevented by the inclusion of adequate casein, sodium bicarbonate, urea or sodium chloride in the diet.

The anatomical and physiological lesions reported to have been induced in rats by feeding sulfonamides in purified diets are listed in Table I.

TABLE I  
*Lesions Induced in Rats by Feeding Sulfonamides in Purified Diets*

Lesion	References
Diminution of growth rate . . . . .	(30, 33)
Increase in prothrombin time of plasma . . . . .	(34)
Hemorrhages . . . . .	(36)
Increase in clotting time of whole blood . . . . .	(42)
Dermatitis and alopecia . . . . .	(36)
Hemorrhagic necrosis of adrenals . . . . .	(40)
Alopecia* . . . . .	(40)
Porphyrin-staining of fur and whiskers . . . . .	(40)
Hyaline necrosis and calcification of voluntary muscles . . . . .	(36)
Leukopenia . . . . .	(35)
Granulocytopenia . . . . .	(35)
Anemia . . . . .	(35)
Hypocellularity of bone-marrow . . . . .	(35)
Achromotrichia . . . . .	(38)
Porphyrin-caked whiskers* . . . . .	(39)
Alopecia* . . . . .	(11)
Hyaline sclerosis and calcification of blood vessels . . . . .	(32)
Necrosis of heart muscle . . . . .	(36)
Cardiac aneurysm . . . . .	(37)
Liver damage (hydropic degeneration of hepatic cells) . . . . .	(36, 37)
Hypertrophy and hyperemia of thyroid . . . . .	(31)
Kidney damage . . . . .	(93)

\* Repetition because more than one factor reported to be involved.

Following the enumeration and description of these lesions, investigations in the various laboratories have developed along two lines; first, the identification of the deficiencies involved and to some extent a more complete clinical and physiological study of the deficiency diseases and, second, an investigation of the mode of action of the sulfonamides in producing the deficiencies.

We will first consider in greater detail the nature of the deficiencies which have been produced.

## II. IDENTIFICATION OF DEFICIENCIES

## 1. Vitamin K Deficiency

Among the vitamin deficiencies caused by the administration of sulfonamides in purified diets, the first to be identified was that of vitamin K. In the first report on this subject, Black, McKibbin, and Elvehjem (30) recorded the observation that the administration of sulfaguanidine did not lengthen the clotting time of whole blood. Black, Overman, Elvehjem, and Link (34) were able to demonstrate, however, that the reduction in growth of young rats receiving this drug was accompanied by a state of hypoprothrombinemia, as measured by the prothrombin time of 12.5 per cent plasma. This effect on the prothrombin level of the blood was counteracted by vitamin K. Of interest, also, is the observation of Black, *et al.* (30), that both effects of the drug could be prevented by the administration either of *p*-aminobenzoic acid or of a liver fraction. The active component of this fraction appeared to be distinct from *p*-aminobenzoic acid and was shown to possess properties which corresponded very closely to those of the "norit eluate factor" ("folic acid"). Welch and Wright (44) noted occasional spontaneous bleeding in their rats receiving sulfasuxidine and observed, also, that this drug caused an increase in prothrombin time similar to that caused by sulfaguanidine. They reported that the effect on the prothrombin level could be largely overcome by a grass juice powder rich in vitamin K or by crystalline biotin plus a "folic acid" concentrate. Day, Wakim, Krider, and O'Banion (45) reported corroborative results concerning the effect of sulfasuxidine on the prothrombin time. They found that cecectomy was helpful in producing vitamin K deficiency, especially in conjunction with the administration of the sulfonamide, and that *p*-aminobenzoic acid partially counteracted the effect of the drug.

Kornberg, Daft, and Sebrell (42) presented evidence indicating that the bleeding originally observed by Daft, *et al.* (36) was the result of a deficiency of vitamin K. By the use of sulfapyrazine, sulfadiazine, and sulfathiazole, they succeeded in producing, rapidly and consistently, so severe a hypoprothrombinemia that it manifested itself by multiple massive hemorrhages and by a marked prolongation of the clotting time of whole blood. The order of effectiveness of the drugs used appeared to be as follows: sulfapyrazine most effective, sulfadiazine and sulfathiazole next, and sulfanilamide, sulfasuxidine and sulfaguanidine least effective. The type of casein in the diet as well as the nature of the sulfonamide administered was demonstrated to play a part in the rapidity of the production of vitamin K deficiency and its severity. *p*-Aminobenzoic acid was shown to counteract the effect of sulfadiazine in producing deficiency signs (43). The administration of crystalline biotin and crystalline *L. casei* factor ("folic acid") on the other hand, appeared to have little or no effect (42).

## 2. Biotin Deficiency

The typical alopecia and dermatitis of "egg-white injury" was adequately described some years ago by Boas (46). Du Vigneaud, Melville, György, and Rose (47) found that this condition could be treated successfully with crystalline biotin. Daft, Ashburn, and Sebrell (36) noted the appearance of this typical syndrome, previously observed in rats only after the feeding of unheated egg-white, in animals given sulfaguanidine or sulfasuxidine in purified diets. Remission of symptoms occurred following the oral or parenteral administration of crystalline biotin in daily doses of 0.5 to 10  $\mu$ g. for 14 days. These findings were confirmed in all essentials by the independent observations of Nielsen and Elvehjem (48) and of Martin (38). Further corroborative data were later presented by other investigators.

## 3. Pantothenic Acid Deficiency

West, Jefferson, and Rivera (40) are among the few investigators who have employed diets containing sulfapyridine. Their dietary regime differed from that of other workers, also, in that yeast, at a level of 0.4 g. per rat per day, was given as a source of the B-vitamins. Deficiency signs, consisting of hemorrhagic necrosis of the adrenals, alopecia, and rusty discoloration of the fur and whiskers (presumably by porphyrin) developed in animals receiving the drug while control rats receiving a similar diet but no sulfapyridine developed no lesions. Animals with deficiency signs were successfully treated by increasing the yeast supplement or by giving an individual daily supplement of 1 mg. of calcium pantothenate. The authors concluded that they had induced a pantothenic acid deficiency in their rats by means of sulfapyridine.

It is of interest in this connection that other investigators have noted similar deficiency signs which were not preventable or curable by pantothenic acid. Martin (38) and Wright and Welch (39) observed achromotrichia in rats receiving sulfaguanidine or sulfasuxidine and adequate pantothenic acid. The latter investigators also noted porphyrin-caked whiskers in their animals. Remission of both signs followed therapy with biotin and concentrates of *L. casei* factor ("folic acid"). Endicott, Kornberg, and Daft (49) observed that rats given sulfadiazine, sulfathiazole, or sulfanilamide and 200  $\mu$ g. of pantothenic acid daily developed adrenal necrosis with or without hemorrhage. No prophylactic or therapeutic tests of *L. casei* factor or biotin were made. From these various data it might be concluded that the achromotrichia, porphyrin-caked whiskers, and adrenal necrosis observed in these experiments are not specific signs of pantothenic acid deficiency. Martin so interpreted his results. Wright and Welch, however, reported that their animals, even when given excessive pantothenic acid, had extremely low hepatic levels of this vitamin unless they were given, also,



biotin and a "folic acid" concentrate. They interpreted these results as indicating that the achromotrichia and porphyrin-caked whiskers which develop in these animals are actually signs of pantothenic acid deficiency, and that they result not from a dietary lack of this vitamin but from a failure in its utilization. In their view, biotin and "folic acid" are essential for the proper utilization of pantothenic acid.

#### 4. *Vitamin E Deficiency*

The sulfaguanidine- and sulfasuxidine-containing diets used by Daft, Ashburn, and Sebrell (36) with which they obtained hyalinization, necrosis, and calcification of voluntary muscle were known to be marginal in vitamin E. They contained 2 per cent of cod liver oil and 3 per cent of Wesson oil and no other fat. As was pointed out by Ashburn, Daft, Endicott, and Sebrell (37), however, control rats receiving similar diets without the drug only occasionally showed a hyalinized or necrotic muscle fiber. It was to be presumed, therefore, that these lesions resulted or were greatly intensified by the administration of sulfonamides. Daft, Endicott, Ashburn, and Sebrell (50), using diets containing lard as the sole fat, with or without sulfasuxidine, showed that a high incidence of lesions resulted from the administration of the drug (9 of 12 animals as compared to 0 of 12 not receiving sulfasuxidine) and that a weekly oral supplement of 3 mg. of  $\alpha$ -tocopherol prevented the appearance of the lesions. It is uncertain at the time that this is written whether or not the apparently obvious conclusion that a vitamin E deficiency was induced will need to be modified in the light of the findings of Milhorat and Bartels (51). These investigators have reported that the substance active in reducing the creatinuria in patients with progressive muscular dystrophy is a combination of inositol with  $\alpha$ -tocopherol. They have indicated that this compound is normally synthesized from the two vitamins in the stomach.

#### 5. *L. Casei Factor ("Folic Acid") Deficiency*

Considerable confusion exists at the present time concerning this newest member of the vitamin B family, both in regard to its physiological properties and in regard to nomenclature. This confusion has arisen largely because of two circumstances, first that this vitamin exists in nature in several closely related forms, and second, that as a result of the limited availability of the pure material, most of the experimental work to date has been carried out with concentrates. Before describing the effects of *L. casei* factor on animals receiving sulfonamides, it appears desirable to present a discussion of nomenclature and a summary of certain pertinent data concerning this vitamin.

There is evidence for the existence of at least five compounds having simi-

lar physiological properties, three of which have been obtained in crystalline form. Table II summarizes information concerning the biological activities of these five substances. There is included in the table, also, reference to a sixth compound which on the basis of present knowledge appears to be so distantly related to the others as to justify its being placed in a separate category.

Three names are commonly applied to one or more member of this group of compounds; the *L. casei* factor, folic acid and vitamin B<sub>9</sub>.

"Vitamin B<sub>9</sub>". Hogan and Parrott (52) in 1939 described an anemia in chicks due to a dietary deficiency of an unknown factor. They named this factor "vitamin B<sub>9</sub>" (53). This annotation was particularly unusual in that the subscript "c" indicated the chick. O'Dell and Hogan (54) later presented a technique for the assay of the antianemia factor and discussed its chemical

TABLE II  
*Biological Activities of L. casei Factors*

Source of Compound	Activity for			
	<i>L. casei</i>	<i>S. lactis</i>	Rat	Chick
Liver*	100%	100%	Yes	Yes
Yeast*	100%	50%	Yes	Yes
Fermentation*	85%	6%	Yes	Yes
Spinach	100%	100%	Yes	Yes
Yeast	<1%	<1%	Yes	—
Unannounced*	<0.001%	100%	No	No

\* Have been obtained in crystalline form.

properties. Its isolation from liver in crystalline form was announced by Pfaffner and coworkers (55) in 1943. These investigators retained the name "vitamin B<sub>9</sub>". They reported that the crystalline material stimulated the growth of *L. casei* and stated "it appears probable that the chick anti-anemia factor, Peterson's 'eluate factor' and William's 'folic acid' factor are the same."

"*L. casei* factor" ("Eluate factor", "Norite eluate factor"). Snell and Peterson (56) reported in 1940 that, among the substances required by *Lactobacillus casei*, was one found in yeast, liver and other natural materials, which could be adsorbed on norite and eluted therefrom with ammoniacal alcohol. They referred to this substance as the "eluate factor", and described methods for its concentration. Stokstad (57) and Hutchings, Bohonos, and Peterson (58) carried the concentration somewhat further. Hutchings, *et al.* also described chemical properties of the factor and showed that a concentrate stimulated the growth of several strains of lactic acid

bacteria. Hutchings, Bohonos, Hegsted, Elvehjem, and Peterson (59) in 1941 showed that a similar concentrate had growth activity for the chick. Mills, Briggs, Elvehjem, and Hart (60) corroborated this finding and reported that their norite eluate preparation also had antianemia activity for the chick. They pointed out similarities in chemical properties between the "norite eluate factor", "factor U" of Stokstad and Manning (61), the alcohol precipitate factor of Schumacher, Heuser, and Norris (62) and Hogan and Parrott's antianemia factor ("vitamin B<sub>6</sub>"). In 1943, Stokstad (63) announced the isolation of one crystalline preparation of the "norite eluate factor" from liver and another from yeast. The two preparations seemed to be closely related but not identical. Stokstad stated that the compound obtained from liver appeared to be the same as that prepared by Pfiffner, *et al.* ("vitamin B<sub>6</sub>"). The following year, Hutchings, Stokstad, Bohonos, and Slobodkin (64) announced the isolation in crystalline form of a third closely related compound. It has been stated that this substance was obtained by a fermentation procedure (65). They modified their previous nomenclature and referred to these three compounds as "*L. casei* factors." On the basis of absorption spectra data, they concluded that none of them could be identical with the "folic acid" prepared by Mitchell and coworkers (66) from spinach.

"*Folic acid.*" Mitchell, Snell, and Williams (66) in 1941 reported that they had obtained from spinach in nearly pure form an acid nutritive which they named "folic acid." *Streptococcus lactis* R was used as the test organism in the purification procedures. Mitchell, *et al.* did not define "folic acid" but they described their concentrate as having similar activity for *L. casei* (and *L. delbrückii*) and for *S. lactis*. The name "folic acid" is euphonious and was quickly and almost universally adopted by workers in the field as a generic term for all substances with similar physiological properties whether derived from liver, yeast, spinach, or other natural materials. In 1944, however, Mitchell, Snell, and Williams (67) defined "folic acid" as a growth factor for *Streptococcus lactis* R. As will appear, this definition destroys the general usefulness of the term. "Folic acid" from spinach has apparently not been obtained in crystalline form.

Stewart, Daft, and Sebrell (68) have presented evidence for the existence in yeast of another substance closely related to the three "*L. casei* factors" which have been mentioned. This fourth "*L. casei* factor" is active for the rat but has little or no activity for *L. casei* or *S. lactis* R. A concentrate of this material was rendered active for these microorganisms by treatment with acid or alkali.

Keresztesy, Rickes, and Stokes (69) announced the isolation of a substance which was less than 1/100,000 as active for *L. casei* as for *S. lactis*.

This compound, called factor SLR, is inactive for the rat (70) and chick (71) which differentiates it sharply from the other five substances we have mentioned. Stokes, Keresztesy, and Foster (72) have presented evidence which indicates that *S. lactis* and all other strains of lactic acid bacteria which can utilize factor SLR, are able to do so because they can convert it to one of the "*L. casei* factors" or a similar active compound.

According to the definition of Mitchell and coworkers (67), factor SLR, despite its lack of activity for the rat, for the chick and for *L. casei* is "folic acid." Activity for *S. lactis* under defined conditions is their sole criterion. By the same definition, the compound of Stewart, *et al.* (68) is not "folic acid" and the fermentation product of Stokstad and coworkers (64) barely qualifies. It appears obvious therefore, that the term "folic acid" must be redefined or must be used in a very restricted sense (in fairness to the originators of the name, it should be noted that they have never suggested its use as a generic term). The name "vitamin B<sub>9</sub>" has the great disadvantage that it introduces the confusing practice of using alphabetical subscripts indicating species. For these reasons, and to conform to the terminology of at least one other laboratory currently active in the field, we have adopted the not altogether satisfactory term "*L. casei* factor" of Hutchings, *et al.* We use it in this review as a general term indicating any substance having activity for the rat and chick, and either active for *L. casei* and *S. lactis* or capable of being changed to an active form by treatment with acid, alkali, or an enzyme preparation such as that described by Mims, Totter, and Day (73).

It appears that the isolation of the *L. casei* factor has made possible the successful culmination of several independent lines of investigation. This newest member of the vitamin B complex was recently announced (74) to be identical with vitamin M, a factor needed by the monkey to prevent nutritional cytopenia (75, 76). There is evidence that it is also the factor U of Stokstad and Manning (61)<sup>2</sup> and it has been identified (78) as a guinea-pig factor (GPF 1) (79). A concentrate has been said to contain a growth factor for mice (80). It is possible also that the panmyelophthisis of György and coworkers (81) is related in some way to a deficiency of the *L. casei* factor. In addition, data have been reported by Watson, Sebrell, McKelvey and Daft which suggest that concentrates of this vitamin may have therapeutic activity in patients with X-ray leukopenia (82). These authors reported negative results in cases of refractory anemia. Sharp, Vonder Heide, and Wolter (83), on the other hand, reported an appreciable increase in the

<sup>2</sup> Chicks on a diet very similar to the one previously used for the development of factor U deficiency (61) respond by an increase in weight to the administration of *L. casei* factor from liver (77).

hematocrit and slight changes in other erythropoietic phenomena following treatment of patients with refractory anemia with an *L. casei* factor ("vitamin B<sub>6</sub>") concentrate.

In 1942, Spicer, Daft, Sebrell, and Ashburn (35) reported the development of leukopenia, granulocytopenia, and anemia, accompanied by a hypocellularity of bone-marrow, in rats given sulfaguanidine or sulfasuxidine. It was noted that the blood dyscrasias could be prevented or corrected by concentrates prepared from liver, which were known to contain the *L. casei* factor. These findings were corroborated by Axelrod, Gross, Bosse, and Swingle (84), by Ransone and Elvehjem (85) and others. Kornberg, Daft, and Sebrell (86) reported the development of similar lesions in rats given sulfadiazine, sulfathiazole, or sulfanilamide. The leukopenia, granulocytopenia, and anemia were again corrected by certain liver concentrates which were known to contain the *L. casei* factor. No positive identification of the active principle in the concentrates could, of course, be made until it was obtained in a pure state. Following the isolation of "vitamin B<sub>6</sub>" by Pfiffner, *et al.* (55) and of fermentation *L. casei* factor by Hutchings, *et al.* (64) we were privileged to test these crystalline materials in rats made granulocytopenic or anemic by sulfaguanidine or sulfasuxidine. As reported by Daft and Sebrell (87), both compounds were found to be active in restoring the level of cells in circulating blood and in permitting the animals to increase in weight.

Concentrates of the *L. casei* factor have been reported to possess additional activities for rats given sulfenamides. Black, *et al.* (34) found that concentrates of this factor prevented the development of hypoprothrombinemia in rats receiving sulfaguanidine. Welch and Wright (44), using sulfasuxidine, made similar observations. Kornberg, *et al.* (42) on the other hand, found no effect of crystalline *L. casei* factor on the hypoprothrombinemia induced in rats by sulfadiazine.

Martin (38) reported that certain concentrations containing this vitamin possessed chromotrichial activity for rats which had become gray while receiving diets containing sulfaguanidine. These animals had received adequate pantothenic acid throughout the experiment and were receiving biotin during the period of therapy with *L. casei* factor concentrates. Wright and Welch (39), using sulfasuxidine, presented corroborative data and reported further that similar concentrates in conjunction with biotin prevented the development of porphyrin-caked whiskers in their sulfasuxidine-fed animals and also caused an increase in the pantothenic acid content of the liver. The reported increase was from a level characteristic of pantothenic acid deficiency to a normal level. As discussed in a previous section, Martin interpreted his results as indicating that the *L. casei* factor ("folic acid") is a chromotrichial factor while Wright and Welch interpreted their data

and those of Martin as indicating that the *L. casei* factor and biotin are needed for the proper utilization of pantothenic acid.

### 6. Inositol Deficiency

Nielsen and Black (41) have reported that rats given sulfasuxidine in a so-called synthetic diet, with supplements of thiamine, riboflavin, pyridoxin, and pantothenic acid and with or without additional supplements of biotin and an *L. casei* factor concentrate, developed a symmetrical alopecia. Inositol supplementation prevented the onset of this condition and animals receiving it grew better and "had a more tidy appearance." No reports confirmatory of these observations have as yet appeared.

### 7. Unidentified Deficiencies

The hyaline sclerosis and calcification of blood vessels (32), the probably related necrosis of heart muscle (36) with, occasionally, bulging of the wall at the apex suggesting beginning or actual cardiac aneurysm (37), and the hydropic swelling of liver cells (36, 37) have not been identified as related to a deficiency of any known vitamin. These changes have not been observed in rats receiving whole dried liver or certain liver extracts which suggests, although it does not conclusively prove, that dietary deficiencies were involved. If we are dealing here with deficiencies of vitamins, the following appear to have been absolved (36); thiamine, riboflavin, pyridoxin, pantothenic acid, nicotinic acid, choline, and biotin.

### 8. Other Lesions

a. *Thyroid and Related Changes.* Mackenzie, Mackenzie, and McCollum reported in 1941 (31) that rats given sulfaguanidine at a level of 1 or 2 per cent in a purified diet developed hypertrophied and hyperemic thyroids. Histologically, the glands showed marked hyperplasia with a great diminution of colloid, and an increase in height of the thyroid epithelium. The administration of yeast, of *p*-aminobenzoic acid, or of excess iodine did not prevent these changes, and Mackenzie and Mackenzie (88) found that they could be obtained equally well with a drug-containing stock diet. These investigators, in addition to sulfaguanidine, employed sulfadiazine, sulfathiazole, sulfapyridine, sulfanilamide, and thiourea with similar results. In a later publication, Mackenzie and Mackenzie (89) reported that: (1) sulfaguanidine definitely depressed the basal metabolic rate of rats, (2) this drug produced an alteration of the cellular pattern of the anterior pituitary including a progressive increase in the number of so-called thyroidectomy cells, (3) the presence of the hypophysis was essential for the production of the thyroid lesions by sulfaguanidine, and (4) all of these changes could be prevented by the administration of thyroxin. They reported further that the

thyroid hyperemia and hyperplasia could be produced in the mouse and dog as well as in the rat. The list of active sulfonamides and thioureas was somewhat enlarged and it was noted that thiourea was about 8 times more active than sulfaguanidine at the same dietary level and that sulfanilamide was less active than sulfaguanidine, sulfadiazine, or sulfapyridine. The authors concluded, concerning the mechanism of action of the drugs, that the primary effect was a depression of the rate of formation of colloid, that a lowering of the BMR resulted and that the thyroid hyperplasia was a reflection of increased pituitary action resulting from this depression.

Astwood, Sullivan, Bissell, and Tyslowitz (90) reported very similar results and reached somewhat similar conclusions. These investigators found sulfadiazine to be by far the most active sulfonamide tested although less active than thiourea. They postulated that a single hypophyseal factor is concerned with both hyperplasia and oversecretion of the thyroid. The sequence of events they consider to be as follows: First, inability of the organism to synthesize thyroid hormone at a normal rate, with consequent reduction in the amount of circulating hormone; second, a resultant production of excess thyrotropin by the pituitary which stimulates the thyroid to hyperplasia and to the release of the thyroid hormone stored therein; and, third, a reduction of the BMR due to exhaustion of the stored thyroid hormone plus reduction in its rate of synthesis.

Franklin and Chaikoff (91) have presented evidence which indicates that the mode of action of the sulfonamides in preventing the synthesis of thyroid hormone is by interfering directly with the conversion of inorganic iodide to diiodotyrosine and thyroxine. By means of *in vitro* experiments with radioactive iodine as indicator, they were able to demonstrate that sulfonamides do not interfere with the capacity of surviving thyroid slices to remove iodine from Ringer's solution but do interfere with its introduction into the benzene ring.

*b. Kidney Lesions.* There is an extensive literature dealing with renal lesions following the administration to experimental animals or to man of various of the sulfonamides. Such lesions have not been reported from sulfanilamide and they are comparatively rare from sulfaguanidine and sulfasuxidine but are not infrequent from many others of this family of drugs. The occurrence of these lesions is frequently explained as due to mechanical injury by the passage through the kidney of relatively insoluble conjugates such as the acetyl derivatives. These derivatives as a rule are more soluble in an alkaline than in an acid or neutral medium, and on this basis sodium bicarbonate is frequently administered clinically with the sulfonamides.

Recent work, although it has supported the point of view that sodium bicarbonate tends to prevent the occurrence of kidney lesions, has thrown

considerable new light on the mechanism whereby this is effected. In addition to sodium bicarbonate, several other substances such as urea (92, 93), sodium chloride (93), and other salts (93), and a high protein diet (93) have been found to exert a preventive effect on the development of the lesions. It has been demonstrated, also, that the passage of various substances through the kidneys non-specifically facilitates the clearance of the sulfonamides (94-96). Kornberg, Endicott, Daft, and Sebrell (93) have shown that the incidence and severity of kidney lesions resulting from the administration of sulfadiazine show an excellent correlation with the amount of conjugated drug deposited in the kidneys, even though the free drug is less soluble than the acetylated derivative, the usual conjugated form.

It is well-known that as a general rule the rate at which a given sulfonamide is administered determines or profoundly influences its level in the blood. It appears most probable also that for a given amount of drug, administered at a given rate, the length of time it circulates in the blood (of which the blood level is a rough measure) in turn influences very considerably the fraction of the drug which is acetylated or otherwise conjugated.<sup>3</sup> There is evidence, further, that the amount of conjugated drug which the animal must dispose of in a given length of time determines to a considerable extent the amount deposited in the kidneys. Finally, as noted above there is an excellent correlation between the amount of conjugated drug deposited in the kidneys and the degree of damage which these organs have sustained. It is obvious, therefore, that there are several points at which bicarbonate, urea, and sodium chloride might intervene to prevent renal damage by the sulfonamides. They might, conceivably interfere with absorption; they might lower the blood level by increasing the rate of excretion of the free drug and thus reduce the likelihood of conjugation; they might interfere in some way with conjugation; or they might facilitate the passage of conjugated drug through the kidneys, by increasing its solubility or otherwise, and in this way prevent or minimize the damage to these organs.

Kornberg and coworkers (93) have presented evidence which indicates that urea and various salts, given at high levels, do not interfere with absorption of sulfonamides but do cause a marked decrease in the level of drug in the blood. A probable explanation of this action has been presented by Peters and coworkers (94, 96) and by Earle (95). These authors suggest that certain of these substances increase the rate of renal clearance of the free drug. The results of Kornberg, *et al.* suggest also that sodium bicarbonate has the further effect of decreasing the damage inflicted by the passage of a

<sup>3</sup> It should be noted in this connection, however, that the data of Kornberg, *et al.* (93) indicate that the blood level of the drug cannot be the only factor which influences the degree of conjugation for there is a marked individual variation between animals receiving identical treatment and showing similar blood levels.



given amount of conjugated drug through the kidneys. It appears probable that this effect depends on the increased alkalinity of the urine caused by the sodium bicarbonate and the increased solubility therein of the conjugated drug.

### III. MECHANISM OF PRODUCTION OF VITAMIN DEFICIENCIES

As has been noted, sulfonamides were introduced into experimental diets in the hope that these drugs would reduce the number of intestinal organisms that the synthesis of vitamins by such organisms would thereby be decreased and that this would lead to the production of new or more severe vitamin deficiencies. The achievement of the anticipated vitamin deficiencies has been abundantly realized. That the mechanism of production of these deficiencies is as predicted is accepted apparently without question by most investigators. There is truly a considerable amount of experimental evidence which supports this assumption particularly in the case of the deficiency of vitamin K, where the body of supporting data is quite impressive. Furthermore, no alternative theory has received any experimental support. On the other hand, there are a few puzzling facts which must be explained before this postulated mechanism can be accepted without reservation as being generally applicable.

In favor of this mechanism are the following observations:

1) Coliform organisms have the proven ability to synthesize a number of vitamins (25).

2) The administration of sulfonamides to rats causes a reduction in the number of these organisms in the animals' gastro-intestinal tracts (97).

3) This reduction in the number of coliform organisms is accompanied by a diminution in the intestinal synthesis of various vitamins (as measured by the amounts found in feces).<sup>4</sup>

4) This is followed by the appearance in the host animals of deficiency diseases. Among these diseases are several which are curable by vitamins which have been shown to be present in the feces in diminished amounts. Examples: vitamin K (34), biotin (36), *L. casei* factor (87).

5) *p*-Aminobenzoic acid, which inhibits the bacteriostatic action of sulfonamides, also prevents the development of vitamin deficiencies in the animals receiving these drugs (34, 99).

In the case of vitamin K deficiency there is the following further evidence:

6) The cecal contents of rats with vitamin K deficiency produced by sulfonamides show little vitamin K activity as compared to the cecal contents of control animals (43).

<sup>4</sup> This has been shown for vitamin K by Kornberg, *et al.* (43). The work of Light and coworkers (98) indicated that the administration of sulfonamides led to a decrease in feces of unidentified growth factors which, from other work (38, 48, 87), were probably biotin and the *L. casei* factor.

TABLE III  
*Lesions and Vitamins Implicated*

Vitamin implicated	Lesions	References
(Used successfully in prophylactic or therapeutic experiments)		
K	Increase in prothrombin time of plasma Hemorrhages Increase in clotting time of whole blood	(34, 42, 44, 45) (36, 42, 44) (42)
Biotin	Dermatitis and alopecia	(36, 48)
Pantothenic acid	Hemorrhagic necrosis of adrenals Alopecia Porphyrin-staining of fur and whiskers	(40) (40) (40)
E	Hyaline necrosis and calcification of voluntary muscles	(36, 50)
<i>L. casei</i> factor	Leukopenia Granulocytopenia Anemia Hypocellularity of bone-marrow Achromotrichia Porphyrin-caked whiskers	(35, 84, 85, 86, 87) (35, 84, 86, 87) (35, 84, 86, 87) (35, 37, 49) (38, 39) (39)
Inositol	Alopecia	(41)
Unidentified	Hyaline sclerosis and calcification of blood vessels Necrosis of heart muscle Cardiac aneurysm Liver damage	(32, 36, 37, 49) (36, 37) (37) (36, 37, 49)
No vitamin involved	Hypertrophy and hyperemia of thyroid Kidney damage	(31, 88-90) (92-96)

7) The production of vitamin K deficiency by sulfadiazine, whether administered orally or parenterally, is related to the concentration of the drug in the cecal contents (43).

8) Similarly, the antagonistic effect of *p*-aminobenzoic acid appears to be

associated with the presence of this substance in the cecum, even when it is administered parenterally (43).

9) Cecectomy increases the incidence of vitamin K deficiency in rats fed diets containing sulfasuxidine (45).

10) The order of effectiveness of the sulfonamides in producing a deficiency of vitamin K (in rats) (42) approximates the order of their bacteriostatic potency against intestinal coliform organisms (in mice) (100).

11) Rats showing an acute deficiency of vitamin K following sulfonamide administration show no lack of ability to absorb and utilize the vitamin. No evidence of increased requirements of the animals for this vitamin has been found (42).

It will be noted from the foregoing that the direct evidence is almost but not quite complete for the production of vitamin deficiencies through the anti-bacterial activity of the sulfonamides. It can hardly be denied that the sulfonamides do inhibit vitamin synthesis by intestinal bacteria. It seems almost certain also that this synthesis would have benefited the host animals. Finally, the inhibition of synthesis was followed by the appearance of vitamin deficiencies. We cannot say with certainty, however, that the inhibition *caused* the deficiencies to appear. It is conceivable that some other effect of the sulfonamides is the critical factor or at least a contributory one. It may be necessary to reduce or eliminate the bacterial population in the intestines by a method other than the action of drugs in order to settle this point to our complete satisfaction.

Of perhaps more immediate interest are three observations which appear at this writing to be difficult to reconcile with the proposed mechanism.

1) There is no evidence to indicate that vitamin E can be synthesized by intestinal bacteria. This vitamin is not normally present in feces (101) and does not appear to be synthesized by rumen microorganisms (102).

2) The order of bacteriostatic potency of the sulfonamides does not agree with their order of effectiveness in producing vitamin E deficiency, biotin deficiency, *L. casei* factor deficiency, arterial lesions, heart lesions, or hydropic degeneration of liver cells (36, 49, 103).

3) In experimental animals, *p*-aminobenzoic acid readily antagonizes the effects of sulfadiazine (43) but much less readily the effects of sulfasuxidine (33). In bacteriostatic tests, on the other hand, *p*-aminobenzoic acid inhibits the effects of sulfasuxidine much more easily than those of sulfadiazine (104, 105).

It cannot be said that these objections serve to discredit or disprove the theory. It is possible that each may be met through more extensive investigations. The recent announcement of Milhorat and Bartels (51), for example, that the biologically effective form of vitamin E is a compound with inositol, may have a bearing on the correct explanation for the development

of muscle lesions in rats receiving sulfonamides even though these lesions are preventable with  $\alpha$ -tocopherol. As to the order of effectiveness of the various sulfonamides in producing deficiency signs in experimental animals, it is conceivable that sulfadiazine and other members of the group have been displaced from their correct positions by their known effectiveness in reducing the basal metabolism. Alternately, it is possible that organisms not belonging to the coliform group may be involved and that in their case there exists a different order of bacteriostatic potency of the sulfonamides. In this connection it will be recalled that Woolley (23) has attributed the intestinal synthesis of inositol to non-coliform organisms. The objection most difficult to meet at the present time appears to be the unexpected resistance of sulfasuxidine to *p*-aminobenzoic acid in the dietary experiments. All of these points appear worthy of further investigation.

#### IV. CONCLUSION

The significance of the results of these investigations cannot be properly evaluated as yet but we know that they have proved of interest to workers in several fields. To the clinician they have suggested that some of the so-called toxic effects of the sulfonamides may have a dietary origin or aspect and that they may be amenable to treatment, either prophylactically or therapeutically, with specific nutrients. To the pharmacologist they have reemphasized the importance of diet in studies of the toxicology and pharmacology of drugs. In the field of bacteriology they have reawakened interest in what we may from the teleological point of view call the function of the intestinal bacteria or more broadly, in the influence of these bacteria for weal or woe on the host animal. To the investigator in the field of nutrition the use of drugs, particularly of the sulfonamides, in experimental diets has proved a most valuable tool. From the list of signs which have developed in animals receiving these drugs and which are as yet unascrivable to a deficiency of any known vitamin it appears that the future for this type of experimentation holds much promise.

It is perhaps unwise to attempt to predict the future course of scientific investigations. From the present vantage point, however, one is tempted to suggest that these researches will eventually be regarded as a part of a much greater field of experimentation, one which concerns the dietary necessities of an individual subjected to the stress of some unusual insult—in this case one of pharmacological nature.

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# Manifestations of Prenatal Nutritional Deficiency

By JOSEF WARKANY

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## I. INTRODUCTION

The development of the zygote into a multicellular and highly differentiated organism requires oxygen and a great variety of building materials which are supplied by the outside world. A growing embryo or fetus depends upon a continuous flow of supplies; interruption of the communication with the outside world leads to arrest of development or death of the developing organism. While this statement holds true for all classes of animals, the supply problem is solved in different ways in the various classes (1, 2). In this article only disturbances of prenatal mammalian nutrition will be discussed.



Inadequate nutrition of the embryo or fetus may have many causes such as faulty implantation of the ovum, disease of the placenta, interruption of the umbilical blood flow, transmission of toxic substances through the placenta and maternal dietary deficiencies. Only the last cause is to be considered in the following pages.

## II. EFFECT OF MATERNAL INANITION UPON THE PRENATAL DEVELOPMENT OF THE OFFSPRING

The fate of the mammalian fetus in an underfed or starving mother has long been a subject of speculation and experimentation. The older literature on the subject was reviewed by Jackson (3), who mentions a number of studies in which underfeeding of the mother resulted in normal development of the fetuses (4, 5) while in other experiments, a reduced birth weight of the young was found (6). Such contradictory results are easily explained. The various investigators used different species of animals; the inanition was of different degrees and commenced at different stages of reproductive life. The diets employed before or during gestation were hardly comparable. More recent experiments point to a definite influence of maternal inanition upon the prenatal development of the young. A thorough study of the effects of inanition in the pregnant albino rat was made by Barry (7). It was found that starvation instituted shortly after copulation resulted in resorption. Starvation begun after the eleventh day of gestation permitted continuation of pregnancy and in the majority of cases the gestation period was prolonged. There were a few absorptions but no premature deliveries. About one third of the offspring were stillborn and the average number of young per litter was reduced. The average weight of the young born of mothers starved during the last half of pregnancy was 40 per cent below the normal birth weight. There was no constant relation between the percentage loss in weight of the mothers and the weight of the newborn. The various organs of the fetus reacted differently to starvation of the mother. The spleen, eyeballs, epididymides, testes, and brain manifested a fairly normal growth during prenatal inanition, while the suprarenals, liver, lungs, thyroid, and thymus showed a marked retardation in growth. Macomber (8) studied the influence of food restriction upon reproduction of the rat. Like others (9, 10, 11) he found that underfeeding led to suppression of the estrus cycle. When the total amount of food consumed by normal female rats was reduced 30 per cent, the interval between periods of estrus was prolonged or the cycles ceased. Only one of 10 females could be made pregnant and 6 young were born which were underweight.

Jonen (12) found in fasting experiments on rabbits that the embryos died soon when the period of starvation began within the first few days of pregnancy. If fasting began in the second half of pregnancy, premature

delivery occurred ten to twelve days later. The fetuses were smaller and lighter than those of control mothers and their cartilage and bone showed definite changes.

Poorly nourished cows may bear calves of normal size for the breed, according to Eckles (13). This author believes that the nutrition of the cow during gestation does not influence the size of the calf at birth except under the most extreme conditions continued for a long time or in case of lack of some indispensable constituent.

The literature concerning the effect of maternal inanition upon the human fetus is hard to evaluate. Many studies were made during or after World War I in which the birth weights of infants before and after the food shortage were compared. According to Jackson (3) who reviewed the literature on this subject no significant differences were found in Austria, England, or Germany. In Russia, on the other hand, where conditions of famine could be observed in certain districts, many authors reported a reduction in the weight of newly born infants during the critical years. Gerschenson (14) states that in obstetrical hospitals in Odessa, the percentage of mature infants with a lowered birth weight (2500-3000 g.) was twice as high in the years of famine (1921-1922) as in the years of prosperity (1924-1926). Since this study excluded stillborn and premature children it does not express fully the effects of famine on prenatal development. Ivanovsky (15) remarks in a report on the physical modifications of the population of Russia under famine: "The number of births decreased enormously, while on the other hand the number of children either prematurely or still-born, monsters, and children with different anomalies, considerably increased."

In the 19th century, and probably even before, many attempts were made to reduce the birth weight of the infant by underfeeding the pregnant mother, the purpose of this procedure being to facilitate delivery (3). The question was revived when Prochownik (16) suggested qualitative dietary changes in place of the quantitative restrictions previously practiced. A diet high in protein and limited in carbohydrates and water was recommended in cases of contracted pelvis. Such diets prescribed by many clinicians were considered ineffective by Bondi (17). Today they are of historic interest only (18).

Thus it would appear that a moderate reduction of the maternal food intake does not materially influence the fetus. However, recent studies in man based on a better knowledge of nutritional requirements and evaluated by statistical methods have shed new light on the influence of maternal diet on fetal development. They have shown that quantitative and qualitative factors of the maternal diet affect the condition of the infant at birth without causing "inanition" of the mother. These studies will be discussed later in a special section (p. 91).

### III. EFFECT OF MATERNAL PROTEIN DEFICIENCY UPON THE PRENATAL DEVELOPMENT OF THE OFFSPRING

The protein requirement for reproduction varies in different species. In the rat a dietary protein content of 5 per cent or less in the diet was found insufficient for ovulation and reproduction (19, 20). Macomber (21), however, stated that the reduction in the amount of protein had little effect upon conception and pregnancy beyond a reduction in fertility, and a slight effect upon the weight of the young even when the reduction was extreme. There is good evidence that qualitative protein deficiency interferes with reproduction, since diets containing wheat (9) or gliadin (22, 23) as the main sources of protein lead to cessation of estrus cycles. Addition of casein or lysine restores ability to reproduce.

In the rabbit very severe protein restriction has little effect upon estrus and ovulation although the animal may lose as much as 25 per cent of its initial body weight (24). The reports concerning the effects of low protein diets upon the prenatal development of pigs are contradictory (25, 26). In sheep fertility is not seriously impaired by diets low in protein, but those low in phosphorus and protein are unfavorable (27). Diets poor in protein affect pregnant mares, but not the development of the fetus (28).

### IV. EFFECT OF MATERNAL FAT AND CARBOHYDRATE DEFICIENCY UPON THE PRENATAL DEVELOPMENT OF THE OFFSPRING

Less than 50 per cent of female rats fed a completely fat-free diet show regular ovulation (29). If the ovulating females are bred to normal males they may gain weight and produce litters. The young are underweight and of reduced viability. The reproductive capacity of rats showing the fat-deficiency syndrome can be restored by a few drops of corn oil, olive oil, and the like. Linolenic or linoleic acid (30), and also arachidonic acid (31) prevent and cure the fat deficiency syndrome. There is no proof that withdrawal of carbohydrate interferes with normal reproduction in the rat (9).

### V. EFFECT OF MATERNAL MINERAL DEFICIENCY UPON THE PRENATAL DEVELOPMENT OF THE OFFSPRING

#### 1. Calcium

Extreme calcium deficiency produced by a diet containing about 0.01 per cent calcium manifests itself in retarded growth, decreased food consumption, reduced activity and sensitivity, prostration, paralysis, and a reduced span of life (32). Although there are no reports available concerning the reproduction of such animals, it can be assumed that their general weakness does not permit mating.

There is little proof that moderate calcium deficiency interferes with

pregnancy. Older statements that female rats fed relatively low-calcium diets did not breed can now be explained by the fact that these diets were deficient in the vitamin B-complex. Macomber (33) found that a maternal diet low in calcium (but supplemented by yeast and cod liver oil) affected the fertility only by increasing the intra-uterine mortality. However, the young which were carried to term were found to be normal at birth. According to this author, pregnancy in the rat makes relatively small demands on the mother's calcium stores, since the young are largely cartilaginous at birth. If, however, rats are held for several generations to a diet low in calcium, the skeletons of the young become progressively more slender and the ribs in the animals of the fourth generation become fragile and suffer spontaneous fractures (34). The fact that pregnancy in the rat can be terminated successfully on a maternal diet low in calcium does not mean that an adequate calcium supply is unnecessary for satisfactory reproduction. It has been shown by Sherman and Campbell (35) that a diet adequate in the sense that it maintained normal growth and successful reproduction could be improved by an increase of its calcium content. The females on an increased calcium intake showed a longer period of ability to reproduce, bore more young and reared a higher percentage of them. Low-calcium diets likewise fail to interfere with successful termination of pregnancy in sows (36); the effect of the deficiency requires several generations to manifest itself (25). A ration very low in calcium and resulting in a decline in the calcium content of the blood plasma, does not interfere with reproduction in cows (37).

## 2. Phosphorus

A review of the studies dealing with the effect of phosphorus deficiency upon reproduction can be found in communications by Eckles, *et al.* (38) and by Friedman and Turner (24). The interpretation of many reports in this field is difficult, since the diets employed were often deficient in several nutritional factors, particularly in protein. The effects of phosphorus deficiency on reproduction in dairy cows and rats can be summarized as follows (38): Uncomplicated phosphorus deficiency in mature dairy cows produced experimentally and continued for two to three years does not cause abnormal estrus but reduces the breeding efficiency, since sterility and abortions are observed under such conditions. In rats fed a diet containing 0.1 per cent phosphorus, (Ca/P ratio between 2.8 and 3.8 and cod liver oil added) Eckles and coworkers (38) observed the following types of reproductive failures: Complete cessation of estrus sometimes following a successful pregnancy or successful lactation; regular estrus cycles but no normal breeding; resorptions in utero; irregular cycles but normal breeding; undersized, weak litters, some of which grew slowly and were successfully weaned. A diet extremely low in phosphorus (0.017 per cent) resulted in marked

skeletal rarefaction and rickets. When the factor of inanition was excluded by paired feeding, no other characteristic change was found. Ovulation took place in about one half of the females fed such a diet (39). The animals were very small. Although reports concerning reproduction were not available, it can be assumed that such rats are unable to mate.

### 3. *Calcium:Phosphorus Ratio*

A careful and extensive study was made by Cox and Imboden (40, 41) concerning the rôle of calcium and phosphorus in determining reproductive success in rats. A Ca/P ratio of 1.0 at a calcium level of 0.49 per cent was thought to be the ideal mineral level and ratio for successful gestation and lactation. At a constant phosphorus intake of 0.245 per cent, increasing the calcium content of the mother's diet from 0.017 to 0.490 per cent improved the reproductive success. In spite of the great differences in the mineral content of the diets used in these experiments, the chemical composition of the young was within the range observed in analyses of stock rats, and the averages were practically identical. The maternal organism regulates obviously the mineral elements supplied to the fetus—within certain limits. A Ca/P ratio of 5.0 in the maternal diet resulted after several pregnancies in a lowered ash content of the bones. The "diet R" employed by Warkany (42) to induce congenital changes in the skeleton of rats which may be termed "fetal rickets" had an approximate Ca/P ratio of 6.0 (p. 89).

The findings of Cox and Imboden (40, 41) were confirmed by Bodansky and Duff (43) who found that the normal calcification of the fetal skeleton depends not only upon the maternal mineral intake but also upon normal function of the parathyroid glands of the mother.

### 4. *Sodium*

Restriction of rats to a diet containing only 0.002 per cent sodium but adequate amounts of other nutritive essentials results in delayed sexual maturity and in disturbances of the estrus cycle which resemble those of vitamin A deficiency (44). Mating of 12 sodium-depleted females with normal males resulted in 2 pregnancies. On autopsy in one female 5 fetuses were found and in another female only one fetus. These fetuses appeared normal but small.

### 5. *Potassium*

Female rats raised on diets extremely low in potassium grow slowly and show delayed sexual maturity and ovulation. When mated with stock males no impregnations occur (45).

### 6. *Manganese*

Diets low in manganese permit sexual maturation and ovulation in female rats. If females reared on such diets are mated with normal males, they give birth to litters of normal size but do not suckle their young. When placed with normal females some of the young can be raised, but they remain small and inferior to the controls (46). Such young are apparently abnormal and have some congenital debility (47). The effect of manganese deprivation needs further study. Skeletal changes have been described in such offspring, but the observations were made on such a small number of animals (48) that evaluation is difficult.

### 7. *Copper*

Ewes fed a diet deficient in copper may give birth to abnormal lambs (49, 50). The newborn lambs are unable to stand to obtain milk from their dams. Those that can get up sway and collapse; their hind quarters seem paralyzed ("Swayback"). The brains of the affected animals show areas of demyelination in the white matter, which may be of microscopic size or appear as a gross cavity. In the spinal cord secondary degeneration of the motor tracts has been found (51). The incidence of this disease can be greatly reduced among the offspring, when the ewes receive salt licks with copper supplements.

### 8. *Iodine*

Iodine deficiency during pregnancy results in the birth of stillborn or defective offspring (52). Manifestations of prenatal iodine deficiency have been described in horses, cattle, pigs, and lambs. The thyroid of the offspring is enlarged, hyperemic, and poor in iodine. According to Smith (53) 1,000,000 pigs were lost in 1917 because of prenatal iodine insufficiency. Such pigs are born hairless and their skin is myxedematous. The gestation period is somewhat prolonged and the young are born full size. The hoofs are underdeveloped and brittle. The heart has a persistent foramen ovale. Smith showed that these defects can be prevented by the administration of potassium iodide or of thyroid gland of sheep.

• In rats goiter can be produced experimentally on diets deficient in iodine. Remington (54) has devised a goitrogenic diet which permits raising of females to maturity. When mated such rats produce a normal number of young which have hypertrophic and hyperplastic thyroids but show no signs of debility. Nelson and Warkany (55) have interbred such goitrous rats for five generations without observing any signs of injury to the young.

Prenatal iodine deficiency occurs in domestic animals in many areas of

the Northern and Northwestern States. It is of great interest that the corresponding condition in man—endemic cretinism—apparently does not occur in the United States, although endemic goiter is prevalent in many sections. However, in certain districts of Switzerland, Austria, and in many other mountainous districts of Europe and Asia endemic cretinism is frequently encountered (56, 57). It is one of the few well-recognized pathologic manifestations of prenatal dietary deficiency occurring in human beings. Endemic cretins are usually born of goitrous mothers. In contrast to athyroid (sporadic) cretins, they usually show at birth an enlarged thyroid gland which is poor in colloid and iodine. Many infants with congenital goiter die soon after birth, since the compressed trachea interferes with respiration. In the children who survive, degenerative processes in the thyroid result in atrophy of the glandular tissue and in hypothyroidism. The symptoms of endemic cretinism consist in dwarfism, retarded ossification, myxedema, deafness, delay in sexual maturation, and mental deficiency. Endemic goiter disappears when the population is supplied with iodine by the addition of potassium iodide to the salt or water. It is hoped that endemic cretinism can also be abolished by these prophylactic measures.

### 9. Iron

When female rats are fed diets deficient in iron, the iron content of the young is reduced to one half of normal, and if the same female is kept on such a diet the following litter will consist of young that have only one fourth of the normal amount of body iron (58, 59). It has been stated by several investigators (60, 61, 62, 63) that female mice and rats kept on a diet deficient in iron for several months produce offspring that develop anemia after birth. According to Fetzer (64) rabbits fed a diet deficient in iron can supply from their own body the iron necessary for fetal growth only up to a limit beyond which fetal death occurs. Infants born to women suffering from hypochromic anemia exhibit a normal blood picture at birth but develop moderate to severe degrees of anemia during the first year of life. This form of anemia may be prevented by administration of iron to the mothers during pregnancy or may be corrected by administration of iron to the anemic infants (65). A form of congenital nutritional anemia has been described by Parsons and Hawksley (66).

•

## VI. EFFECT OF MATERNAL VITAMIN DEFICIENCY UPON THE PRENATAL DEVELOPMENT OF THE OFFSPRING

### 1. Vitamin A

Vitamin A deficiency results in a change of the vaginal smears of female rats. There appears an increased number of cornified cells, while the leu-

cocytes become sparse until the cyclic changes are replaced entirely by the phase of keratinized cells (9, 67). Estrus and ovulation continue in such animals as indicated by their frequent matings, but fertilization or implantation often fail or resorption takes place (68, 69). At certain levels of deficiency a part of the matings may result in litters (70). A thorough study of the gestation period in vitamin-A-deficient rats was made by Mason (71, 72). At levels of vitamin A deficiency necessary to induce vaginal cornification but insufficient to produce xerophthalmia, resorption of all or of part of the fetuses occurs during early pregnancy. These early fetal deaths are caused by disturbances of the fetal nutritive supply resulting from placental injury. Localized areas of infection, leucocytic infiltration, and cellular necrosis are observed at the junction of the ectoplacenta and the maternal decidua (71, 72, 73). When the fetuses fail to undergo early resorption their growth is often retarded and they die during late pregnancy, as a result of placental damage. In such cases gestation is frequently prolonged (74) because of a disturbance of the birth mechanism. Growth of the fetuses varies, some being undersized, others excessively large. Many young are born dead and the majority of those born alive die within a few days after delivery. Some of those which live through to the weaning stage have xerophthalmia.

H. Mellanby (75) studied the effect of maternal dietary deficiency of vitamin A on dental tissues in rats. From the age of 3 to 4 weeks female rats were fed the deficient diet for different periods before experimental breeding was begun. The young of mothers who had been on the diet 24 to 25 weeks prior to the birth of their litters showed gross dental abnormalities soon after birth. Their incisor teeth were very abnormal in shape, the teeth were retarded in development and the molars were misshapen. There were also changes in the mothers' teeth but in all experiments the abnormalities were more pronounced in the young than in their mothers.

In cattle and in sheep estrus and impregnation are not so much affected by vitamin A deficiency, but gestation is disturbed; the birth of dead or weak calves with or without eye lesions and suffering from severe diarrhea has been reported (76). The eye lesions which are seen particularly in immature animals simulate infectious keratitis. In sheep deficient in vitamin A the delivery of dead or non-viable lambs has been reported (77). In pigs the estrus cycle becomes irregular, being more frequent and of longer duration than normal. Breeding during the early stages of deficiency results either in abortions or in the birth of dead pigs at farrowing time (78, 79).

The congenital blindness of the offspring of vitamin-A-deficient mothers deserves special discussion. Congenital blindness in the offspring of poorly nourished animals has often been reported. Early communications were neither specific in the statement of the deficient nutritional element nor in the analysis of the eye defects. According to Moore, Huffman, and Duncan



(80) two types of congenital blindness must be distinguished: the "true vitamin A type" of blindness, which is obviously a severe form of xerophthalmia (71, 76, 81, 82), and another type of blindness which is associated with a constriction of the optic nerve (83-89). The latter type was difficult to explain at the time of the publications and Moore, *et al.* state: "It seems doubtful that vitamin A could be concerned with such bone malformations as in the cases reported in this paper, but such an explanation might be plausible, if the absence of vitamin A, in some indirect way, raised the intracranial pressure." In a later paper, however, Moore (88) established a relationship between carotene and this type of blindness in calves. Since then Wolbach and Bessey (90) have demonstrated that the nervous lesions of vitamin A deficiency are caused by a disproportionate growth of the central nervous system in relation to the surrounding bone and the constriction of the optic foramen can now be considered also a "true" manifestation of vitamin A deficiency. Davis and Madsen (91) made quantitative studies of carotene intake of cattle and of plasma levels of carotene and vitamin A in heifers during pregnancy. Cattle with a plasma carotene level of 25  $\mu\text{g.}$  and a vitamin A level of 16  $\mu\text{g.}$  per 100 ml. usually show no signs of vitamin A deficiency (88, 92). However, higher levels are required for normal reproduction. Heifers with plasma carotene levels from 30 to 60  $\mu\text{g.}$  and vitamin A levels from 10 to 20  $\mu\text{g.}$  per 100 ml. had calves that were either dead or weak and blind. The calves of heifers with carotene levels of 78, 96, 143  $\mu\text{g.}$  and vitamin A levels of 22 and 24  $\mu\text{g.}$  per 100 ml. were of good quality.

The manifestations of prenatal vitamin A deficiency described by Hale (93-95) were of an entirely different type. This investigator fed gilts of known stocks a diet consisting of white kafr, cottonseed meal, limestone, and salt. This deficient diet was given for 150 to 200 days before breeding and during the first 30 days of gestation when the formation of the eye is completed in the pig. After this stage the diet was supplemented by cod liver oil in order to make successful termination of pregnancy possible. The offspring of sows treated in this way were blind owing to anophthalmos or microphthalmos. They showed also other malformations, such as accessory ears, cleft lip and palate, subcutaneous cysts, and misplaced kidneys. Sows fed the same experimental diet but supplemented by cod liver oil or green fodder farrowed normal pigs. The occasional appearance of congenital malformations in the offspring of sows fed a diet deficient in vitamin A had been described before (96), but Hale deserves credit for proving in classic experiments that genetic factors can be ruled out as the cause of the malformations (95). From a nutritional point of view the experiments need further interpretation, since the diet used was probably deficient in several factors. Hale recognized clearly the importance of the maternal diet in the early stages of pregnancy "when so many of the vital organs of the embryo are being formed."

Hale (95) questioned whether congenital blindness due to maternal vitamin A deficiency could be induced in rats. Cannon (97) enumerates a number of authors who investigated reproduction in vitamin-A-depleted rats and failed to discover congenital malformations in the offspring. He also reported failure to induce congenital anomalies by maternal vitamin A depletion in the young of rats.

Warkany and Schraffenberger (98) controlled the vitamin A intake of female rats from the time of weaning by feeding a diet deficient in vitamin A and by adding 25  $\mu$ g. of carotene every tenth day. Such animals obtained



Fig. 1. A. Section of Eye of Normal Newborn Rat

B. Section of Eye of Abnormal Newborn Rat, Offspring of Vitamin-A-Deficient Mother

The iris, ciliary body and anterior chamber not developed. Cleft in the retina through which connective tissue enters the eye.

enough carotene for growth and maturation but apparently were unable to store vitamin A. As soon as regular cycles were established, these females were placed on a purified diet entirely free of carotene and vitamin A and then were bred. They developed signs of vitamin A deficiency within a few days and only very few were able to carry their offspring to or near term. The few young obtained showed either external abnormalities of the eyes or defects discoverable by histologic examination. Fig. 1B shows such an abnormal eye. In this section the anterior chamber, the iris, and the ciliary body are absent. The vitreous has not formed and in its place is connective tissue which enters the eye through a cleft in the lower half of the retina. Below this cleft the retina is everted. This is typical of coloboma

retinae, which represents a malformation of the eye caused by arrested development in an early embryonic period. The retina is completely disorganized and resembles cerebral cortex more than retinal tissue. At the present time only the sections of the eyes have been thoroughly studied but it can be said that other soft tissues as well as the skeleton show signs of arrested development. Abnormalities of the eyes have been recognized in embryos of such vitamin-A-depleted rats as early as the 16th day of prenatal life.

## *2. Vitamin B*

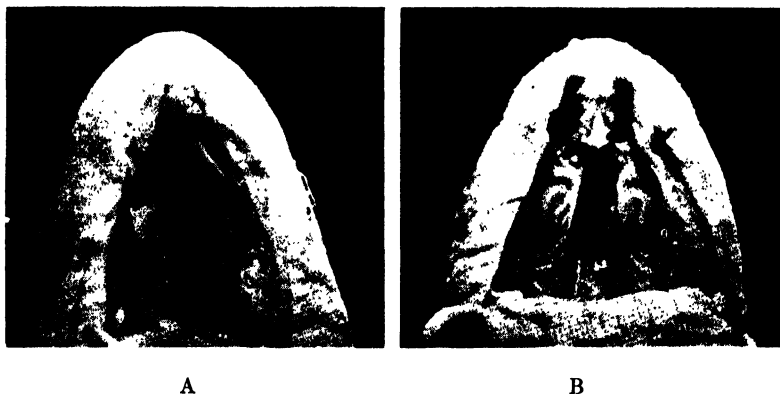
Before the B complex was subdivided into the various B vitamins known at the present time, Evans and Bishop (9) showed that absence of "vitamin B" from the diet resulted in disturbance of the normal estrus cycle of rats. It was shown later that the absence of either thiamin (99) or riboflavin (100) from the diet results in anestrus. In rats in which insemination is obtained before deficiency symptoms become severe, intra-uterine resorption, early interruption of pregnancy, prolongation of gestation, difficulty in parturition, and occasional delivery of small and weak young have been observed (101). In rabbits miscarriage occurs as a rule if an absolute lack of vitamin B exists in the diet during the first half of pregnancy. The same deficiency in the second half of pregnancy results in delivery of inferior young (102).

Beriberi in infants is usually considered to be the result of breast-feeding by a mother who has latent or manifest B<sub>1</sub> deficiency. However, several cases have been observed in infants so soon after birth that the existence of congenital beriberi seems established (103, 104, 105). In a case described by van Gelder and Darby (106) the mother who had been on an inadequate diet for 4 months preceding delivery did not exhibit any marked clinical findings of beriberi. The infant was born cyanotic and almost aphonic, with extreme tachycardia and a greatly enlarged heart. When the infant was 42 hours old an initial dose of 50 mg. thiamin was administered and this treatment was repeated several times. Eighteen hours after the first administration of thiamin the infant was dramatically improved. Urinary thiamin assays and dextrose-, pyruvate- and lactate-blood levels, in the fasting stage and following ingestion of dextrose, indicated a state of thiamin deficiency in the mother. It is possible that such cases occur more frequently in children but are unrecognized. In India (107) and Japan (108) stillbirths and premature deliveries are frequently the result of maternal B<sub>1</sub> deficiency and the infant mortality in these countries is severely affected by the maternal dietary habits. A tendency to hemorrhage in the newborn infants of mothers deficient in vitamin B has been described (109, 110).

It has been mentioned that a lack of riboflavin in the diet of rats may

result in anestrus (99); in such rats, cycles can be restored by giving riboflavin unless the deficiency has lasted about 10 weeks. Then the damage becomes irreparable. Low levels of riboflavin ( $3 \mu\text{g. per g. of diet}$ ) may permit normal reproduction but in the second generation a decreased ability to withstand deprivation of riboflavin or of thiamin has been noticed (111).

When mature female rats are placed on a diet lacking riboflavin and bred to stock males, they give birth to normal offspring if they conceive immediately. If conception is delayed they may resorb their young or become sterile before pregnancy takes place (112). It is not possible to state how long it takes to induce sterility since individual rats behave differently in this respect. This variability may be caused by differing abilities of the



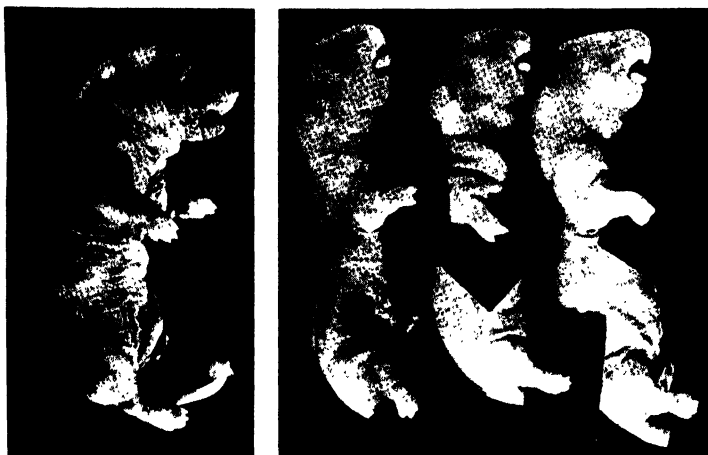
A

B

Fig. 2. A. Normal Palate of Newborn Rat

B. Cleft Palate of Newborn Rat, Offspring of Riboflavin-Deficient Mother

intestinal bacterial flora to synthesize riboflavin. It is of great interest that there exists between normal fertility and sterility a stage in which defective offspring develop. This at least is the interpretation we give to the following experiments. In 1940, it was reported by Warkany and Nelson (113) that female rats reared and bred on a deficient diet gave birth to young one third of which had congenital skeletal malformations. The diet consisted of yellow corn meal, wheat gluten, calcium carbonate, sodium chloride, and viosterol (diet I). On this diet the females were retarded in growth and maturation, but when they were finally bred many became pregnant and a number of them delivered young at term. Some of the young could be recognized as abnormal by external inspection (Fig. 3B-D); in others skeletal defects were manifest only after clearing (Fig. 4B-D). The malformations consisted in shortening of the mandible, tibia, fibula, radius, and ulna, in fusion of ribs, sternal centers of ossification, fingers and toes, and



A

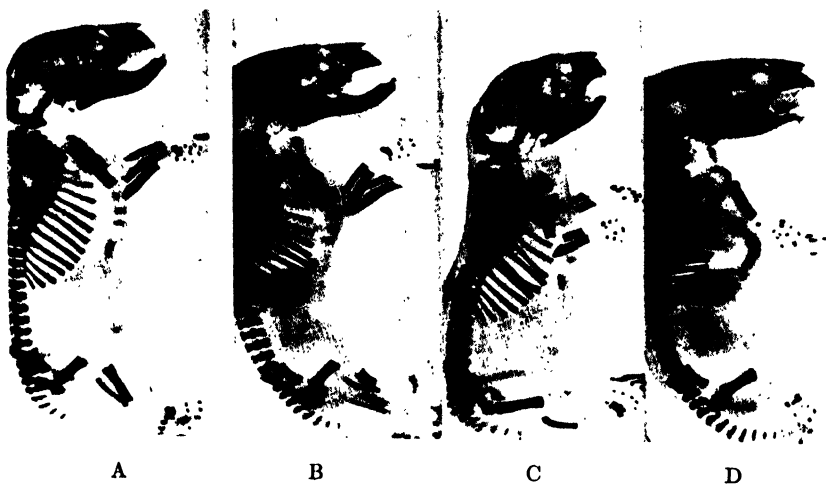
B

C

D

Fig. 3. A. Normal Newborn Rat

B-D. Abnormal Newborn Rats Showing Short Mandibles, Various Degrees of Syndactylism and Clubfoot (B)



A

B

C

D

Fig. 4. A. Cleared Specimen of Normal Newborn Rat

B-D. Cleared Specimens of Abnormal Newborn Rats

Showing shortness of tibia (B); shortness of mandible, radius and ulna and absence of tibia (C); shortness of mandible, absence of radius, tibia and fibula (D).

in cleft palate (Fig. 2B). There were great variations among specimens since the degree of the abnormalities as well as the number of the affected structures varied. In the same litter normal young and young of differing abnormality could be found (114). However, while some bones were more or less often affected, others were always spared. Thus a definite pattern was recognized in these malformations (pattern of diet I). Histologic examination revealed that not only osseous but also cartilaginous structures were deformed. They showed a lack of division in the longitudinal and transverse directions, thus resulting in a reduction in the number of the skeletal elements. The faulty and delayed ossification was considered secondary to the abnormalities of earlier stages of the skeleton (115, 116).

TABLE I  
*Breeding Results on Alternating Diets*

	Offspring	
	Normal	Abnormal
Diet I.....	0	4
Diet I.....	0	4
Diet I + Liver.....	8	0
Diet I.....	6	1
Diet I + Liver.....	10	0
Diet I.....	8	2
Diet I.....	0	7
Diet I + Liver.....	3	0

In order to prove the nutritional origin of these malformations, females of the same strain of rats were bred on an adequate stock diet and others on diet I supplemented by 2% dried liver. The offspring of these females never showed abnormalities of the pattern of diet I (117). When females who had produced abnormal offspring on diet I were changed to diet I plus liver they always had normal offspring. When changed back to diet I their next litter did not always contain abnormal young but subsequent litters usually did. Table I shows the breeding results of a female that was bred eight times with the same male on alternating diets. She produced abnormal or mixed litters when on diet I, and always normal litters when on diet I plus liver.

A search was made for the preventive factor in liver. Addition to the maternal diet of iodine, manganese, liver ash, casein, cod liver oil, and wheat germ oil were not preventive, but a mixture of 5 crystalline vitamins of the B-complex: riboflavin, thiamin, niacin, pyridoxin, and pantothenic acid, prevented the abnormalities. Further experiments showed that in this mixture the riboflavin alone had preventive power (112). This result

was in accord with the fact that diet I is poor and liver rich in riboflavin. It was also shown that on a highly purified maternal diet in which the vitamin-B-complex was represented by crystalline substances, abnormalities of the pattern of diet I were obtained in the offspring, if riboflavin was omitted from the maternal diet. When riboflavin was added no abnormal young were obtained (118). Thus it was shown that the pattern of diet I could be prevented by riboflavin.

Attempts were then made to ascertain in which phase of the development the fate of the affected structures was decided. It was shown that the malformations of diet I could be prevented by liver as late as the thirteenth day of gestation (119). This indicated that the malformations were not determined before the 13th day of prenatal life. Histologic sections showed that the malformations were already present in cartilaginous structures, which indicated that the deviation from the normal development occurred before or at the stage of chondrification (115, 116). Chondrification begins in the rat on the fourteenth and fifteenth days of prenatal life. Thus one can assume that the malformations are determined not before the thirteenth and not after the fifteenth days of gestation. A critical stage exists then in the 13- or 14-day rat embryo in which the presence or absence of sufficient riboflavin is of a decisive influence on the development of the embryo. At about this time most of the affected parts of the skeleton undergo rapid changes (119). Undifferentiated mesenchymal structures develop into the well-differentiated membranous skeletal elements which are the forerunners of the cartilaginous and osseous skeleton. This change from mesenchyme into the membranous skeleton seems to be impaired by a deficiency in riboflavin (112).

Taylor, *et al.* (120) examined the effect of high levels of pantothenic acid on reproduction in the rat and the mouse. The breeding performance of rats and mice fed Purina dog chow, containing 14 mg. of pantothenic acid per gram, was compared with that of animals receiving the same diet and supplements of 100 mg. of calcium pantothenate. There was an increase of 21 to 27 per cent in litter size in the supplemented group as compared to the controls. Weights of the brain and heart in day-old rats were smaller in the supplemented group. On a purified diet free of choline rats can grow to maturity and reproduce (121).

### 3. Vitamin C

Vitamin C depletion in early pregnancy results in abortion or absorption of the fetuses (122, 123), while deprivation during the latter half of pregnancy leads to stillbirths or delivery of premature or weak offspring with scorbutic changes (122). In spite of many reports suggesting that pregnancy has an attenuating effect on scurvy, much of the data is contradictory (72).

The human fetus absorbs ascorbic acid from the placenta (124) and restrictions of women's diet during pregnancy lead occasionally to abortion but not to deprivation of the fetus (125). The fetus seems to draw the vitamin C from the mother as long as appreciable amounts of ascorbic acid are present in the maternal plasma (126). Several studies have shown that the vitamin C content of the blood plasma of the fetus is higher than that of the mother (126, 127, 128, 129). The fetus seems to act parasitically on the mother with respect to vitamin C and vitamin C deficiency is not rare in pregnancy and puerperium (126, 130, 131). In spite of its parasitical action the fetus does not always obtain the required amounts of vitamin C from the mother and congenital scurvy occurs in man (132).

The only species known definitely to require a dietary source of vitamin C are man, monkeys and guinea pigs. It is possible that in mice, rabbits, swine and cattle vitamin C is required under certain conditions (45). A report by Philipps, *et al.* (133) indicates that dietary ascorbic acid is necessary for reproduction of cattle. These authors found that cows which had failed to reproduce conceived after injections of ascorbic acid.

#### 4. Vitamin D

Although an extensive literature exists on congenital rickets, there is little known about the effects of pure maternal vitamin D deficiency upon the development of the fetus. It is well established that in the rat absence of vitamin D from the maternal diet does not affect calcification of the skeleton of the fetus unless the Ca/P ratio assumes extreme values (134, 41). Thus the congenital skeletal changes induced in rats by a maternal rachitogenic diet (42) depend upon a high-calcium and low-phosphorus content as well as upon a lack of vitamin D. Female rats fed a modified Steenbock and Black rachitogenic diet which contains a supplement of 2% dried liver (diet R) produced young 45 per cent of whom had congenital skeletal malformations. Pronounced curving of the radius, ulna, tibia and fibula as well as angulation of the ribs occurred in the offspring (Fig. 5A). It is of interest that the histologic picture of the affected bones was not characteristic of rickets, since there was no abundance of osteoid (Fig. 5B). However, the same diet R resulted in typical rachitic changes in infantile rats. Since a similar observation has been made in cattle (135), it must be assumed that a rachitogenic diet results in different skeletal changes before and after birth.

Lack of vitamin D in infants results in rickets even in the presence of a favorable Ca/P ratio in the diet. A pure prenatal vitamin D deficiency could probably lead to rickets in human fetuses, but it is doubtful whether such cases ever occur. In the instances of congenital rickets reported by Maxwell, *et al.* (136, 137, 138) the maternal diets were deficient in vitamins, minerals and energy.





Fig. 5. A. Cleared Specimen of Abnormal Newborn Rat

Showing curved radius, ulna, tibia and fibula and broadening of the distal parts of the ribs.

B. Section of Proximal Epiphysis of Abnormal Newborn Rat

Showing abundant cartilage and irregular line of ossification (Silver-stain).

These abnormalities can be prevented by supplementing the maternal diet with vitamin D.

### 5. Vitamin E

A lack of vitamin E in the diet results in sterility in the female rat, although the animals appear healthy, show normal growth, normal estrus rhythm, normal ability to conceive and normal implantation (139, 140). Vitamin E is not necessary for the early phases of reproduction. Administration of this vitamin as late as the 5th day after copulation makes possible normal termination of pregnancy (141). Up to the tenth day of gestation the pregnant uterus appears normal on gross examination and serial sections of embryos show also normal development. On the tenth day the pregnant uteri develop a softening of the implantation sites, which show a blue discoloration because of blood in the amniotic cavity. Sections of the embryos reveal rarefaction of the mesenchyme and failure of the blood-forming tissues. There are changes in the ectoplacenta as evidenced by the more com-

pact appearance of the fetal portion and the failure of the mesodermal elements. Necrosis begins on the fetal surface by the 13th day. The decidua basalis retains its normal appearance until it degenerates on the 15th day. By the end of the 21st day only the implantation sites indicate the preceding pregnancy (140).

It is uncertain whether vitamin E is necessary for reproduction of animals other than the rat and mouse (142). Goats and rabbits can reproduce on a vitamin-E-free ration (143). It has been suggested, however, that vitamin E can be useful in the treatment of sterility of other mammals (142, 144) including man (145).

### 6. Vitamin K

Moore, *et al.* (146) reported that female rabbits fed a vitamin-K-deficient diet for 40 days and mated with normal males aborted during the late first or early second trimester of pregnancy (8th to 14th day). Retroplacental hemorrhages were considered responsible for the abortions. The prothrombin levels were lowered in the rabbits but they did not reach a critical level, which suggests an unusual susceptibility of the placenta to deprivation of vitamin K. Abortions occurred again if the females were bred once more while fed the deficient diet, but normal term pregnancies resulted when vitamin K was added to the diet.

It is well established that a prothrombin deficiency exists during the first few days of the neonatal period in man and that administration of vitamin K to the mother before delivery or to the infant increases the prothrombin values (147). But there is little evidence that the hypoprothrombinemia of infants is due to a dietary deficiency of the mother.

## VII. RECENT STUDIES ON THE RELATIONSHIP OF MATERNAL NUTRITION TO FETAL DEVELOPMENT IN MAN

In recent years a number of carefully controlled studies have been undertaken to ascertain whether maternal nutrition influences the course of pregnancy or the development of the fetus. These studies were done on a large scale; the diets were evaluated quantitatively and qualitatively; the infants were carefully examined and the results were statistically analyzed.

A study of the influence of the prenatal diet on the mother and child was made by Ebbs, *et al.* (148). An analysis of the prenatal diets of 400 women with low incomes was made at the beginning of observation, usually at mid-pregnancy. The patients were classified into 3 groups; those receiving a poor diet throughout pregnancy; those having a supplemented diet; and those receiving a good diet. These mothers were observed during the prenatal period, during labor and convalescence. The infants were rated at birth and

at the ages of 6 and 12 months. The additional food supplied to the mothers of the supplemented group consisted of 45 g. protein, 46 g. fat, 60 g. carbohydrate, 840 cal.; 1.45 g. calcium, 15 mg. iron, 50–80 mg. vitamin C, 350–400 I.U. vitamin B, and 2000 I.U. vitamin D. The observation in the Prenatal Clinic averaged four and one half months. There were more cases of anemia, toxemia, and threatened miscarriages in the poor diet group, while the total number of complications in this group was about double that of the supplemented group. There was a definite difference in the fate of the fetuses in the two groups, since in the poor diet group more miscarriages, stillbirths and prematures were observed than in the supplemented and good diet groups. The birth weights of the infants did not seem influenced by the type of the maternal diet. It is also of interest that the incidence of illness in the babies up to the age of 6 months and the number of deaths resulting from these illnesses were many times greater in the poor diet group.

Williams and Fralin (149) studied the diets of 514 pregnant women and found that only 10 diets could be termed good, 209 fair and 295 poor when compared to the recommended dietary allowances of the National Research Council. However, there was no evidence that complications of pregnancy, stillbirths or neonatal deaths could be correlated to dietary deficiencies, except that 84 per cent of a group who presented a history of nausea and vomiting in early pregnancy had an intake below the pregnancy standard of 600 units of vitamin B.

Burke, *et al.* (150–151) studied the influence of the maternal nutrition during pregnancy upon the condition of the infant at birth. The observations on 216 women selected from the prenatal clinics of the Boston Lying-in Hospital, and their infants are reported. The nutritional data were obtained by carefully taking a record of the food intake at various periods, the food likes and dislikes, the amounts purchased and the money spent for food and by various other methods of cross-checking which permitted an accurate evaluation of the maternal diet. Nutritional standards were set up for the rating of the dietary intakes from the fourth through the ninth month of pregnancy. Nutritional essentials such as protein, calcium, phosphorus, iron and the vitamins were rated as excellent, good, fair, poor and very poor; the daily caloric intake was included in the rating. The maternal diets were finally classified as excellent, good, fair, poor and very poor. Only 14 per cent of the women consumed "excellent or good" diets, 17 per cent "fair to good," 29 per cent "fair", 23 per cent "fair to poor" and 17 per cent "poor to very poor" diets. Thus 40 per cent of these women were malnourished during the prenatal period. The infants were studied at birth and during the first 2 weeks of postnatal life. They were classified as "superior," "good,"

"fair," and "poorest." A definite relationship was established between the maternal diet and the condition of the infant (Figs. 6 and 7).

In another publication Burke, *et al.* (152) correlated the protein intake of the mother to the infant's measurements at birth. A significant relationship was found to exist between the protein content of the mother's diet and the birth length of the infant. A similar correlation existed between the mother's protein intake and the birth weights. These authors believe "that less than 75 g. of protein daily during the latter part of pregnancy result

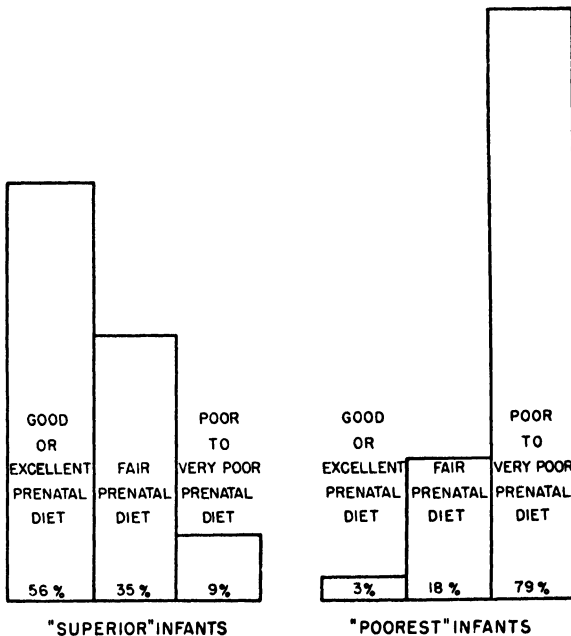


Fig. 6. Nutrition Studies During Pregnancy by Burke, *et al.*, *Am. J. Obstet. Gynecol.* 46, 46 (1943)

in an infant who will tend to be short, light in weight, and most likely to receive a low pediatric rating in other respects."

The Interim Report of the People's League of Health in England on "Nutrition of Expectant and Nursing Mothers" (153) contains data of a large scale nutritional experiment. The investigation which lasted from March 1938 to the end of 1939 was planned to show whether additions of vitamins and minerals would benefit the course of pregnancy and labor and the newborn child. An inquiry into the diet of 5022 expectant mothers

showed there was no well-marked deficiency of first class protein among the women answering the questionnaires. A shortage of calcium was noted in 70 per cent and an iron deficiency in 98 per cent of the diets. As regards vitamins, the deficiency of A was the most common, since more than 50 per cent of the women took less than required. The intake of vitamins B<sub>1</sub> and C were low but not extremely low. One half of these mothers were given the following daily supplements: Saccharated iron carbonate (1.2 g.), equivalent to 0.26 g. ferrous iron, calcium lactate (2 g.) equivalent to 0.26 g. calcium,

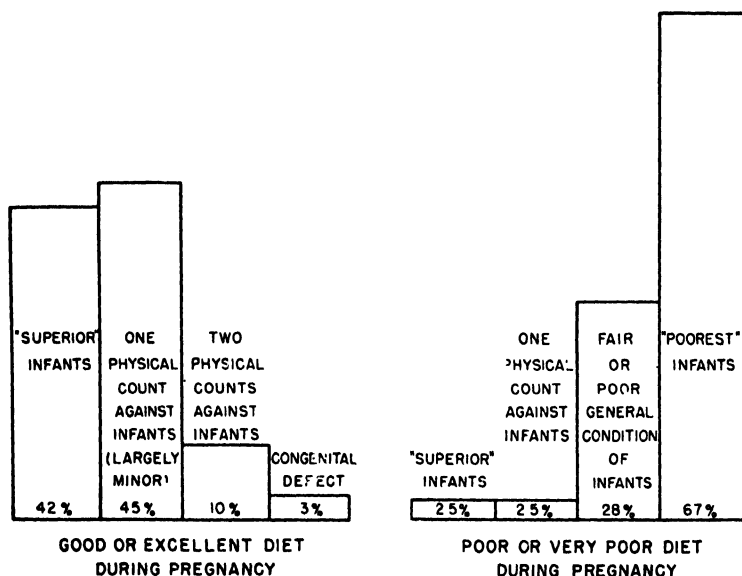


Fig. 7. Nutrition Studies During Pregnancy by Burke, *et. al.*, *Am. J. Obstet. Gynecol.* 46, 47 (1943)

minute quantities of iodine, manganese, and copper, adsorbate of vitamin B<sub>1</sub> containing all factors of the B complex (1 g.), vitamin C (100 mg.), halibut liver oil 0.36 g. (vitamin A 52,000 I.U., and vitamin D 2500 I.U. per g.).

Evidence was found of benefit to the infant from the improved diet of the pregnant mother. The chances of a woman carrying her child to term were improved by the diet. The number of premature children born to the mothers receiving the supplemented diet was significantly lower than that born to mothers who did not receive the supplements. The relatively greater proportion of deliveries at term among the treated women is of importance

in view of the fact that 50 per cent of infantile deaths under one month are due to prematurity.

An experiment of supplementary feeding in pregnancy on a large scale was reported by Balfour (154). The material was well selected and classified as to parity, age and economic conditions. There were 11,618 women who received supplements and 8095 who did not. Both classes received milk in some form. The supplemented group received vitamins A, D, and B-complex, calcium, phosphorus, and iron. There was a significant reduction in the stillbirth and neonatal mortality rates of the fed group as compared with the controls.

The importance of prenatal nutrition for the development of the teeth and jaws is well recognized (155, 156). While earlier studies were concerned chiefly with maternal metabolism of calcium, phosphorus and vitamin D, recent experimental studies (75) direct attention also to other nutritional factors.

### VIII. GENERAL CONSIDERATIONS

The ova of mammals develop within the maternal genital tract. Thus they are well protected from mechanical injuries as well as from nutritional deficiencies. They are as a rule not exposed to fluctuations of the nutritional supplies as are the ova of lower classes, since the maternal tissues serve as a reservoir from which the developing organism can draw the required chemical substances. It is obvious that the protection thus afforded is not unlimited and that starvation, severe disease or death of the mother may also result in death of the ovum. But it was until recently the consensus that the diet of the mother was of no importance to the fetus which was considered a parasite capable of exhausting the maternal stores to the extreme. This is obviously not the case. It can be gathered from the foregoing review that dietary maternal deficiencies result frequently in resorption, abortion, stillbirth, prematurity and weakness of the offspring. It is also shown that congenital malformations of the offspring can be induced by maternal nutritional deficiency. This new knowledge in the field of nutrition deserves special discussion, since it demonstrates the effect of dietary deficiencies upon the earliest processes of growth and differentiation. Little is known about the environmental causes of congenital malformations and these nutritional studies will undoubtedly give a new impetus to research in this field.

It was thought until recently (2) that prenatal mammalian nutrition is an all-or-none phenomenon. It was believed that either the embryo is capable of supplying itself adequately from the mother's tissues or that it will die in case of failure. There is some truth in this conception, but it is not entirely correct. Obviously there exist some borderline deficiencies which

lead to interesting consequences. The discussion of these deficiencies is in part still theoretical, since the critical dietary levels of these borderline states have not been determined but it seems that for vitamin A deficiency they will soon be established (91).

In the case of a borderline riboflavin deficiency the mothers have apparently sufficient amounts for maintenance of the estrus cycles and for gestation. The fetuses are able to grow to almost normal size but certain processes of differentiation are disturbed. A reduction of riboflavin below the critical level leads to sterility or embryonic death, while an increase beyond this level results in the birth of normal young. It is understandable that such a borderline deficiency may lead to the production of normal, abnormal and even mixed litters, since slight variations of the growth or position of the embryos and minor fluctuations of the riboflavin level may decide whether the individual will be normal or abnormal. Moreover a constant diet does not guarantee a constant blood level, since synthesis of riboflavin by the mother's intestinal bacteria and coprophagy may account for significant variations (112).

Stockard (157) has emphasized that the structural response of the embryo to arresting agents is specific for the embryonic stage during which the agents act. Developing organisms go through critical moments, periods of accelerated growth and differentiation in which they manifest a marked sensitivity to injurious factors, while they are relatively immune to the same noxae at other periods of development. It is of interest that Hale (95) as well as Warkany and coworkers (119) were able to establish critical periods in which the manifestations of the respective vitamin deficiencies are determined. An adequate diet after the critical period may make possible successful termination of pregnancy but it does not repair the malformations which have become established. Thus addition of cod liver oil to the diet of the sow 30 days after mating does not prevent congenital eye defects in the pigs; and the pattern of diet I can not be prevented in the offspring when the mother rats receive supplements of liver after the fourteenth day of gestation (p. 88). It can be shown that at the critical period between the thirteenth and fourteenth days of prenatal life rat embryos show a remarkable differentiation of most of the skeletal parts affected in the pattern of diet I (119).

It was concluded that the arrest of development occurs in the membranous skeleton but it remains to be explained why a low level of riboflavin affects only some skeletal structures adversely while others are spared. In this respect the nutritional deficiency behaves like toxic agents for which a differential susceptibility of developing organisms has been repeatedly demonstrated (158). This differential behavior is noted in the pattern of

diet I as a rule in the transverse plane, since the skeletal parts near the mid-dorsal line are normal while the ventrodistally situated ones are more often deformed. Since the proximal and unaffected parts are differentiated before the distal and affected parts, one may conjecture that in a borderline deficiency there may be enough riboflavin for the early development of the membranous skeleton, while a deficiency exists when the later parts are formed.

In the critical period, between the thirteenth and the fourteenth days, the rat embryo weighs only 40 to 112 mg. (159), while at birth deformed young may weigh 4000 to 5000 mg. Since riboflavin is known to be necessary for growth, one may wonder how such marked increase in size and weight is possible in the presence of a riboflavin deficiency. This apparent contradiction can be explained by the assumption that differentiation requires a higher riboflavin level than normal growth. A mutual independence of growth and differentiation is well recognized in experimental embryology (2).

There exists a widespread belief among the laymen as well as among physicians that systemic and multiple congenital malformations are always the result of changes in the "germ-plasma," that is, genetically determined and hereditary. This belief is not correct; in fact it has been shown repeatedly that developmental processes can be altered by environmental disturbances in the same way as by abnormal genes (160). It will be of great interest to the geneticist that types of congenital malformations, which are sometimes genetically determined, such as microphthalmos, cleft palate, brachygnathia and the like, can be produced in the offspring by maternal deficiencies of well defined chemical substances like vitamin A or riboflavin. Since it has been suggested that genes act as enzymes (161) it may be worth while to point out that riboflavin is known to be an essential constituent of a number of important enzyme systems which perform specific functions. Perhaps either a nutritional riboflavin deficiency or a defective gene leads to the same congenital abnormality, because the same enzyme system necessary for the normal development of the skeleton is disturbed. It seems possible that nutritional experiments like those cited above will contribute to the understanding of gene actions. There is no conflict in the results of genetic and nutritional investigations into the causes of malformations.

The postnatal behavior of offspring with congenital malformations has not yet been investigated. Some of the eyeless pigs obtained in the experiments of Hale (95) were used for genetic studies, but no other observations were reported. In the case of riboflavin deficiency the abnormal young could usually not be raised. However, if it becomes possible to produce regularly viable young with defective organs, many branches of experimental medi-



cine will benefit. It should be mentioned in passing that errors in embryonal development have played an important rôle in the theories of the etiology of tumors.

Since animal experiments cited in the foregoing review have shown that dietary maternal deficiencies result often in reproductive failures, it will be asked whether comparable conditions ever occur in man. There can be no doubt that in foreign countries maternal nutritional deficiency is frequently responsible for a high infant mortality (163, 107, 56, 57) and it is probable that similar correlations exist in the United States (150, 151) and Great Britain (148, 153, 154).

Nutritional deficiencies are not limited to the low income groups. Malnutrition may develop in any expectant mother. Nausea, vomiting and perverted appetite are frequent occurrences in pregnancy. Diets restricted in certain nutritional elements are often prescribed and deficiencies of single dietary factors may accidentally develop.

The appearance of the mother does not always betray her nutritional state and in case of deficiency the fetus may suffer more than the mother. It has been shown in animal experiments that a vitamin-A-level sufficient for maternal health may result in disaster to the offspring (91); and vitamin-A-deficiency does not affect the teeth of the mother so severely as those of the young (75). A mother with latent beriberi may give birth to a child with congenital manifestations of the disease (106). Female rats with a riboflavin deficiency so mild that they can undergo repeated pregnancies, often give birth to young who have the most severe malformations. Female animals fed iron-deficient diets without developing anemia produce iron-deficient offspring that develop anemia (60-62). Iodine deficiency which causes only an enlargement of the thyroid in the mother may result in cretinism in the child (56).

These examples will suffice to illustrate that in the struggle for nutritional factors between mother and offspring it is not always the fetus who obtains what he needs. The myth of the fetus as a parasite can be upheld only in regard to vitamin C (126, 127).

The finding of critical periods in the development of the embryo, in which there is unusual susceptibility to nutritional deficiency (93, 119) opens new perspectives in the field of prenatal nutrition. It emphasizes the importance of a satisfactory nutritional state of the mother in the earliest periods of pregnancy. The organogenesis of the human embryo is practically finished ten weeks after conception, at a time when the expectant mother is not accorded any special privileges. Nutritional supplements are as a rule not given before the second half of pregnancy. Thus it may happen that an embryo is injured by nutritional deficiency in the early weeks of prenatal life while the mother appears in a perfect nutritional state at the end of

## PRENATAL NUTRITIONAL DEFICIENCY

pregnancy. Nutritional care and nutrition studies should include the earliest periods of pregnancy as well as the late ones.

Finally it should be mentioned that nutritional deficiencies are not always caused by incomplete diets. A surplus of one nutritional element in the diet may result in the deficiency of another one. The disturbing substance may interfere with the absorption of an indispensable factor (avidin-biotin) or destroy it (thiamin by enzyme of fish) or inhibit it by displacing it from its normal rôle (vitamin inhibitors) (162). Metabolic disorders of the mother or increased demands of the offspring (twins) may in the presence of an adequate diet result in states of nutritional deficiency.

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# Growth Factors in Microbiology

## Some Wider Aspects of Nutritional Studies with Micro-organisms

By B. C. J. G. KNIGHT

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It is not intended that this article shall be an exhaustive record of the present state of knowledge concerning so-called growth factors for micro-organisms. War-time conditions affecting adequate access to the literature make a complete review difficult, the more so since the subject is in rapid growth, with a wealth of new publications continually appearing. The writer has therefore aimed to give a general outline correlating various aspects and to indicate some main trends of development. It is hoped that against this general background will be seen the rapidly-appearing new work which, for one reason or another, is not specifically mentioned here. Conscious omissions are made only for reasons of expediency within the above framework, and the selection of illustrative examples has been made on the same principle. This article will deal mainly with bacterial growth factors, and with cross-links with other nutritional studies.

## I. INTRODUCTION

### *1. The Development of the Study of the Nutrition of Micro-organisms*

A general review of bacterial growth substances is best developed historically in order to show the evolution of some of the generalizations which have emerged, how the differentiation of the special field of *bacterial nutrition* came about, and why this limited viewpoint is no longer so fruitful as the wider perspective now possible. Concurrently, but at first quite separately, there developed also the study of the nutrition of yeasts, beginning effectively with Wildiers' demonstration (420) which initiated the yeast "bios" problem (see Section VII, Biotin). The historical development of this field, directed to the determination of the exact nutrient requirements of yeasts, has been reviewed by Williams (423). Here discussion will be restricted to the bacterial field until the coming together of the two fields showed their underlying unity.

The separate study of bacterial nutrition arose out of the desire to cultivate bacteria *in vitro*. Originally this was a purely technical problem of laboratory practice. The media used were complex and designed to simulate more or less closely the natural environment of the organisms, of which the majority first studied by bacteriologists were those associated with human and animal disease. What may be termed "classical bacteriology" developed in the main by using the laboratory cultivation of organisms on complex media which were considered adequate if the organisms would grow therein.

Knowledge of the chemical composition of the media was not then of importance. The practical problem became more acute as organisms were found which required special media or conditions of cultivation (e.g., gonococcus, meningococcus, pneumococcus, *Hemophilus influenzae*, freshly-isolated tubercle bacillus, Johne's bacillus, etc.). In general the cultivation problem was solved empirically in a manner adequate for the immediate purpose; media were prepared in the laboratory in which most organisms would grow and which allowed the manipulations of bacteriologists to be carried out. The riotous profusion of media which were compounded more or less empirically to satisfy the growth requirements of various organisms testifies to the variety and fastidiousness of bacteria in their nutritional requirements.

Quite early the advantages which would accrue from simple media were recognized, and several workers (e.g., Urichsky, 395) attempted to use media of simple and known chemical composition. Similar attempts were made during the following 30 years. Most of the early claims to have grown pathogenic bacteria in media of this type are now known to be invalid. The growth which was observed was due to the unsuspected presence of very small quantities of essential nutrients ("growth factors") coming from impure organic compounds of natural origin (e.g., glucose, lactate, asparagine) or carried over in the inocula. The smallness of the quantities of these unknown essential nutrients which would permit visible growth was necessarily not appreciated. One school of workers, associated with Braun (44, 45, 46) was successful in cultivating certain organisms of the *Salmonella* group in chemically simple media, many of these organisms having simple nutrient requirements, unlike the majority of organisms associated with animal disease.

Another stimulus to work on the cultivation of bacteria in media of known chemical composition came from a different direction with the discovery of autotrophic bacteria by Winogradsky (434). But the non-pathogenic bacteria did not receive until much later the attention which was lavished on the organisms of human and animal disease.

It is significant that attention first became focussed on the nutrient requirements of those bacteria which are among the most exacting in this respect. This was either because the complex media they required for growth were easily injured in preparation (owing to the presence of essential labile substances) and thus rendered nutritionally deficient, or because unusual nutrient supplements were required. Thus the question of the nutrient requirements of bacteria became differentiated as a particular problem of bacteriology in much the same way as the human and animal vitamin problem forced itself on the attention of biochemists, namely as a problem of nutritional deficiency.

As early as 1912, Twort and Ingram (390, 391) showed that John's bacillus, which would not grow on complex media adequate for other acid-fast bacilli (including *Mycobact. tuberculosis hominis*), would do so if the medium were enriched by incorporation of the bodies of acid-fast bacilli (e.g., *Mycobact. phlei*) which were able to grow on ordinary laboratory media. The reasoning which led them to this deserves quoting in their own words:

"Considering . . . the apparent close relationship between the tubercle bacillus and the bacillus of pseudo-tuberculous enteritis, and the fact that both these bacilli live in the bodies of bovines, we judged it probable that they would require the same chemical substances for building up their protoplasm, certain of which substances could be elaborated from artificial media by the tubercle bacillus but not by the bacillus of pseudo-tuberculous enteritis—in other words, that the latter bacillus has lived a pathogenic existence from such remote ages that it has lost the original power of its wild ancestor—whatever bacillus that may have been—and can no longer build up all its necessary foodstuffs outside the animal body.

It was thought probable that if these substances could be obtained ready formed, and were added to some good artificial medium (Dorset's egg medium) the bacillus would grow, and, further that these substances might be elaborated by allied micro-organisms such as the tubercle bacillus, and even stored up as reserves in their envelopes" (391, pp. 69-70). . . .

"From the fact that from the remotest times the timothy-grass bacillus (*Bacillus phlei*, Moeller) found on *Phleum pratense* must have been continually ingested by ruminants in their food, the possibility of this bacillus being the wild ancestor from which John's bacillus had arisen is at once obvious, and if such be the case one might expect, from what has already been said, that *B. phlei* would be a very suitable variety to use in preparing the medium" (391, p. 77).

These quotations contain in essence a general conception of bacterial nutrition and its wider aspects which the work of succeeding years has developed and enriched. Twort and Ingram attempted to isolate the active substance synthesized by *Myc. phlei* which was required by John's bacillus. They thus made one of the earliest studies in the field of bacterial growth factors. Twort and Ingram's book (391) is certainly one of the few classics in the history of bacterial chemistry.

Not until 1917 was the existence of special growth factors for another bacterial species clearly recognized; these were the X and V factors for *Hemophilus influenzae* (58). Fildes (87) studied the differentiation of the X and V factors required, either together or separately, by various organisms of the *Hemophilus* group, making one of the earliest comparative studies of nutritional requirements within a group of related organisms. Here again the existence of special nutrient requirements became apparent because the lability of the V factor led to its destruction during heat sterilization. These observations were made at a period when, as is clear from the name vitamin

or V factor, the study of human nutrition had led to the recognition of vitamins and to their attempted isolation. From this time onwards the vitamin conceptions of animal studies affected the ideas of workers in bacteriology. Many premature attempts were made with impure animal vitamin preparations to show effects on the growth of micro-organisms. The results were necessarily equivocal for lack of pure vitamins. At the same time it was shown, however, that some species of bacteria, able to grow in media deficient in certain vitamins of the B group, did in fact synthesize some of them, as shown by animal feeding experiments. In 1934, Williams and Roehm (429) showed a marked growth stimulation of certain yeasts by crystalline preparations of natural vitamin B<sub>1</sub>.

### *2. Analysis of Complex Bacteriological Media*

The attack on the problem of bacterial nutrition through the analysis of complex empirical media began in the 1920's, particularly with the work of Mueller (253) who sought to fractionate complex media for growing hemolytic streptococci. Mueller did not succeed in his main object but he did discover a new amino acid, methionine. This was the first discovery of a new compound of general biological importance coming through the study of bacterial nutrition. The contributions of Mueller and his school have been outstanding ever since. Arising out of the need to know what were the electrode-active compounds present in culture media being used for oxidation-reduction potential studies of obligate anaerobic organisms, Fildes and Night (157, 158) found it necessary to study the exact nutritional requirements of various organisms. They also, with others, were led to attack the problem from the analytical side (154).

### *3. Recognition of Physiological Cross-links*

The first clear link between the nutrient requirements of micro-organisms and animals came when vitamin B<sub>1</sub>, thiamin, became available in pure form. Schopfer (330, 331, 332) showed that thiamin was an essential growth factor for the mould *Phycomyces blakesleeanus*, and, later, Tatum, Wood, and Peterson (381) showed it had a marked growth-promoting effect on certain strains of lactic acid and propionic acid bacteria. Since then the number of known growth factors has rapidly increased, and the physiological cross-links between different types of organisms have become clear. The inter-relations between the growth requirements of various classes of organisms and the way in which studies in different fields have been complementary is illustrated in Table I, which lists substances common to the growth requirements of various types of organism. (Consideration of the basal nutrients, carbohydrates, amino acids, salts, is omitted from this table, as from the review as a whole.) It will be seen that the substances

listed in Table I fall into several groups. Thus in Group A, thiamin, riboflavin, and pyridoxin were compounds new to chemistry which were first discovered as a result of their biological activities observed in the nutrition of higher animals. Group B substances were all known to organic chemistry and had been isolated from biological materials before their roles in the nutrition of micro-organisms were observed, or indeed before any special physiological functions for them were known. Group C, pantothenic acid and biotin, were, like Group A, new to chemistry but were first detected through their biological activities in the growth requirement of a micro-organism (yeast). *p*-Aminobenzoic acid is at present in a class by itself in

TABLE I

*Inter-relations in Growth Requirements of Different Classes of Organisms*

Substance:	First recognized as an essential nutrient for:	Subsequently implicated in the nutrition of:	Component of enzyme systems, etc.:
A. Thiamin (vitamin B <sub>1</sub> )	Higher animals	Bacteria, lower fungi, protozoa, roots of higher plants	Co-carboxylase; thiamin—protein enzymes
Riboflavin (vitamin B <sub>2</sub> )	“ “	Bacteria, insects, lower fungi	Several flavoprotein enzymes
Pyridoxin (vitamin B <sub>6</sub> )	“ “	Bacteria, insects, lower fungi, plant roots	{ Tyrosine decarboxylase co-enzyme
B. Nicotinic acid	<i>Staph. aureus</i>	Higher animals, plant roots	{ Co-enzymes 1 and 2
Pyridine nucleotides	<i>Hemophilus influenzae</i>	—	{ Pyridine-protein enzymes
Uracil	<i>Staph. aureus</i> (anaerobic)	—	—
Glutamine	Streptococci	—	—
Pimelic acid	<i>C. diphtheriae</i>	—	? role in biotin biosynthesis
Inositol	Yeasts	Mouse, mold ( <i>Ashbya gossypii</i> )	
$\beta$ -Alanine	Yeasts	<i>C. diphtheriae</i>	Part of pantothenic acid
C. Pantothenic acid	Yeasts	Bacteria, higher animals, insects	—
Biotin	Yeasts	Bacteria, higher animals, insects, lower fungi	—
D. <i>p</i> -Aminobenzoic acid	Bacteria	Higher animals, insects, lower fungi	—

that it is the first compound whose hitherto unsuspected biological activity was discovered, not directly as a growth substance, but indirectly through its neutralization of an artificial growth inhibitor which was interfering, as it now appears, with an essential metabolic reaction. Biotin, whose effect in animal nutrition was first discovered through its reversal of a natural inhibitor, avidin, was independently found as a growth factor for micro-organisms before its effect in animal nutrition was clearly recognized.

*4. Nomenclature, Ideology, Theory*

The history of the subject has now developed to the point where the older nomenclature is often a hindrance, since it has led to sterile discussion

and may hide the real nature of the phenomena which need to be understood. Most of the known growth factors, accessory growth substances, growth promoters, vitamins, nutrilites, etc., as they have been variously named, have been discovered as a result of observation of the results of a nutritional deficiency. The biological test object has grown sub-normally, sub-optimally, pathological conditions have developed or there has been entire failure to grow, and a nutritional deficiency has been recognized. In order to assay the content of biologically active substance in various preparations which can remedy the deficiency condition, suitable biological methods of testing have had to be designed.

In the case of micro-organisms, not only have the substances being sought been highly active per unit weight but the amount needed to cause a biological response is extremely small, since so little actual increase in mass of cells is needed to give an observable effect. Thus, great care needs to be taken in designing the growth response test to avoid effects due to inadvertent contamination with the active substance. These conditions emphasized the smallness of the quantities needed for a given biological response, and thus these substances tended to be grouped as a class separated from the grosser constituents of a diet. Furthermore, in order to obtain clear-cut results in testing it was easier on the whole to look for 'all or nothing' types of response rather than to measure *rates* of growth, as would have been needed to assay substances which could stimulate a growth already taking place slowly. Many known and unknown substances could affect rates of growth, but the new substances being sought, since they had very marked effects, were in many ways the most easy to define in terms of a 'growth' or 'no growth' response. In the earlier analytical studies of culture media much insistence was placed therefore, for practical reasons, on an observation of growth or no growth when an addendum was made to a deficient medium. Obviously the criterion of 'no growth' could be variously defined. But as long as it was sensitive enough to enable a clear course to be steered through the morass of growth accelerators and stimulators, and allowed a clear recognition of the 'active' fractions in chemical fractionation procedures, even visual observation of cultures could be a successful guide. For these practical reasons much emphasis was placed on the occurrence and detection of "essential nutrients," without which the micro-organism could not grow. The definition of growth usually was the formation of a mass of cells of the order of that obtained in classical culture media in the usual growth period (2, 3, 4 days, etc.); and 'no growth' signified usually no visible traces of growth when examined by the naked eye. These criteria have served for the detection and identification of a number of compounds needed for the growth of various micro-organisms. Clearly, however, a more detailed definition of what was meant by 'growth' could be instituted in every assay method. This has been done in much of the more recent work. Various aspects such



as the initiation of germination of spores and of cell-division, increase in amount of cell-material built up, rate of increase of cell substance formed, etc., have been measured to determine the response to addenda to the cultural environment. In this way a more detailed knowledge is being built up of the chemical and physical composition of the environment which determines growth in the general sense. Mueller (264) has well discussed these considerations.

Out of the birth-pangs of the newly developing field of microbial nutrition and metabolism various names were coined to denote as a class the active substances sought. Williams (421) suggested the use of the word *nutrilite* for those organic substances which in minute amounts function for yeast and micro-organisms in a similar manner to the vitamins of animal nutrition. He remarked on the "unfortunate etymological implications" of the word vitamin and was against the undue extension of its use. The name *nutrilite* was unobjectionable from this standpoint, implying only an importance in nutrition and nothing as to the chemical nature of the substance (423).

György, dealing largely with animal nutrition (114), raised some aspects of the problems of nomenclature in discussing "what should be considered a vitamin." He quotes his own previous proposal for a definition: "Vitamins are food constituents of organic origin, minute quantities of which have specific biologic effects and lack of which in the food produces pathologic disturbances, occasionally only under special conditions" (112). György points out that at least two objections can be raised against this definition. Thus, choline is used not in "minute quantities" but in relatively high doses. Should it then be excluded from the group of water-soluble vitamins and be called only a "dietary essential"? (143). If choline be excluded on the ground that it is not used in minute quantities, then also ascorbic acid, nicotinic acid and pantothenic acid should be excluded from the category, since they are all required in relatively large amounts compared with others of the water-soluble group such as biotin, thiamin, riboflavin. Conversely, if choline be considered a vitamin, why not methionine, which has choline-like effects on nutrition, and, for that matter, the essential amino acids as a whole? György further points out that the classification of a vitamin dependent upon its character as a food constituent (as in his former definition) is no longer so valid, since certain vitamins synthesized by bacteria in the digestive tract are certainly used by host animals.

Clearly the vitamin conception as first envisaged, from the point of view of a substance making good a deficiency in a diet, has passed through a series of developmental stages. The category no longer serves any very useful purpose, now that the analytical approach to nutritional requirements has progressed so far and is being widened to a more general metabolic

approach. The substances which were first detected through the effects of their deficiencies were, first, those constituents of an adequate diet which were deficient in abnormal "natural" diets (e.g., the thiamin-deficient polished rice diets which caused beriberi, or ascorbic acid-deficient diets, lacking in fresh fruit and vegetables, causing scurvy), and later, those substances which were deficient in artificial diets compounded on what knowledge of nutritional requirements was available at the time. The major constituents (e.g., protein, carbohydrate, fats) required in a diet being known, the unknowns were those substances required in much smaller quantities which had hitherto escaped attention. But it is now clear that these vitamins do not form a class in any real sense; they appeared to form a class only as long as they were unknown.

When the diets required for the normal growth of lower organisms such as bacteria, yeasts, molds etc. came to be examined, it was hardly possible to study deficiencies of diet in terms of the development of characteristic pathologies; what was observed was the ensemble of phenomena characterizing growth, i.e., multiplication of a small number of cells to the mass of cells of a fully grown culture. The measurement of such growth was analogous to measurement of growth and maintenance of health in higher animals. Because of the much greater velocity of multiplication and of the concomitant building-up of cell material, differences in the rates of these processes are very easily seen with micro-organisms. There is much greater sensitivity to variation in the cultural conditions than with the higher animals. But the ideology of animal nutrition was carried over to a large extent to the field of micro-organisms, and it was largely for this reason that the test for activity of supplements to a deficient culture-medium was so often designed in terms of "growth" or "no growth."

With the progress of the field of microbial nutrition, cases were found where this simplified approach was not so necessary or so fruitful. Mueller (254, 255) for example, began to measure the effects on the growth of *C. diphtheriae* of active supplements added to deficient media by measurement of the actual amounts of cell substance formed. He could thus observe effects in terms of growth curves, which gave a more dynamic picture of the effects of supplements. This type of approach was more flexible in that it could take account of a biological behavior less easily observed with higher organisms, namely adaptability in nutritional requirements, and was also more suited for observing changes in rate of growth.

At the same time the complementary inter-relation between nutritional requirements and the organism's own synthetic abilities was becoming clearer. Once it had been recognized that many of the substances required by one set of organisms were substances synthesized by another set of organisms, the latter thus not requiring those substances as *dietary*

constituents, a more general conception became possible, and it became clear that these substances played an essential part in metabolic processes common to both classes of organism.\*

From the foregoing it is seen how the aspect of the metabolic requirements of micro-organisms (and of higher animals) which first received most study was the nutritional one. On this basis the analysis of diets and nutrient media brought to light the chemical nature of many of the more elusive components of these nutrients. But emphasis on the nutritional aspect tended at first to segregate these more elusive nutrient components as a class, and general names for the class were given, such as vitamin, essential growth factor, etc. When, however, the nutritional question is viewed from the standpoint of metabolism, many of the apparently difficult questions of definition and nomenclature disappear. The substances which an organism takes from its nutrients are used as material for building-up the new cells. These cells carry out a complex interwoven series of processes, which is the life of those cells, and consists in taking compounds from the environment and synthesizing other compounds to make new cells. The extent and rate of multiplication of new cells will depend on the efficiency with which the processes of construction are carried out. This efficiency (here used in the general and not only thermodynamic sense) will clearly depend partly on the availability of the materials of construction. This will in turn depend upon the rates of utilization and synthesis of the various materials of the enzyme systems whose continued functioning is the life of the cells. The fundamental biochemical processes of cell-life—the essential metabolism of the cells—form the cardinal feature, and certain of these processes are common to the widest variety of cell. Where organisms may differ, however, is in the means whereby the materials for these processes are acquired. But here a sharp metaphysical distinction into “acquired from the environment” or “synthesized by the cell” is not possible. For it is clear that a certain rate of synthesis might be too slow to yield a required substance at the required rate. Effectively then the cell would depend upon an external source of supply, to a degree which would be relative to the rate of synthesis of this substance. Hence a given substance, required as a component of one of the essential metabolic processes, might appear in three different roles as a component of the nutrients. It might appear: (1) as an ‘essential’ nutrient, when its rate of synthesis by the cell was so slow as to be insignificant; (2) as a growth stimulant, when its rate of synthesis was somewhat faster but still slow enough to be a limiting factor; or (3) as a substance not required at all for nutrition, because the cell could synthesize it so fast that it was not a limiting factor in growth. It is the metabolic process which is the essential thing and the compounds used in carrying it out are essential metabolites, *i.e.*, the substrates used for the process, or the substances which

\* See footnote, added in proof, p. 228.

form parts (prosthetic groups, etc.) of the enzyme systems which carry out these essential reactions. Fildes (89) suggested the use of the term *essential metabolite* in the above sense. With the more dialectical orientation outlined above, discussions as to whether a given substance is an essential growth factor, vitamin, etc., or not, or whether it is only a growth stimulant, promoter or accelerator, etc., are clearly sterile. What matters is to show how any given substance which affects growth plays its part. And very often it will be found that it has a relation to some essential metabolic process, the role it plays in *nutrition* reflecting the mode by which the cell acquires a sufficient quantity of it at a sufficient rate. Throughout the review the term *essential metabolite* will be used in the above sense, leaving it to the collection of data here presented to support the validity of this viewpoint.

#### 5. The General Implications of Nutritional Studies with Micro-organisms

Table I reflects the way in which studies of the nutrition of micro-organisms have contributed to nutritional studies in general. Broadly there are two reasons for this. The first is the practical one of the ease and rapidity with which large numbers of experiments can be performed. But this alone would be no great advantage if it were not for the underlying unity of all biochemistry, just discussed. If nutritional requirements are seen as a reflection of a given organism's ability to synthesize essential metabolites, it is at once clear that findings concerning the *nutrition* of one organism give information about essential metabolic processes common to many organisms. For reasons which may be at bottom evolutionary, micro-organisms as a whole display the very widest variety of nutritional requirements. This, coupled with the rapidity of experiment, gives the basis for the recent wide developments in the field of nutrition of micro-organisms and their influence on nutritional studies in general.

### II. THIAMIN

Thiamin (Vitamin B<sub>1</sub>) was first shown to be an essential metabolite for a micro-organism in 1934 by Schopfer (330, 331) who found that the fungus *Phycomyces blakesleeana* would grow *in vitro* on a medium of known chemical composition when thiamin was added, but not without it. This was the concluding step in previous work (329) designed to isolate the active material in various natural sources, which had been found a necessary addition to the basal medium before continued growth could take place. When crystalline (natural) vitamin B<sub>1</sub> became available Schopfer found, following a suggestion by Burgeff (332) that this substance might replace the active extracts he had obtained. As early as 1930, Williams and Roehm (429) had observed a marked growth stimulation of certain yeasts by a crystalline preparation of natural vitamin B<sub>1</sub>.

### 1. Thiamin in Bacterial Growth

Thiamin was first implicated in bacterial growth by Tatum, Wood, and Peterson (381) who observed that it had a marked growth-promoting effect on certain strains of propionic acid bacteria when added to a deficient basal medium. Not all the strains of propionic acid bacteria used required to be given thiamin; some strains grew and fermented well on the basal medium alone; still others did not respond to the addition of thiamin alone and evidently required an additional growth factor.

For *Staphylococcus aureus*, thiamin (or its constituent pyrimidine and thiazole components together) was found by Knight to be an essential nutrient (155, 156). Unlike the propionic acid bacteria, all the typical strains of *Staph. aureus* which have been examined (156, 289) appear to need thiamin as an essential nutrient. No observations have been recorded of strains of *Staph. aureus* which can synthesize thiamin, as with the propionic and lactic acid bacteria; such strains may yet be found. O'Kane (273) records strains of *Staph. flavus* which grew in a simple ammonia medium without added growth factors.

Niven and Smiley (272a) report that the intact thiamin molecule was required by 20 different strains of *Streptococcus salivarius*; co-carboxylase (thiamin diphosphate) was 40% more active than thiamin, on a molar basis.

Möller (250) for certain lactic acid bacteria found that thiamin had a stimulating effect on growth. Wood, Andersen, and Werkman (435) had previously included thiamin in the basal medium they used for showing that riboflavin was required by certain lactic acid bacteria. Wood, Geiger, and Werkman (437) using strains of heterofermentative lactic acid bacteria (in Orla-Jensen's classification *Betabacteria*) found that thiamin or riboflavin could replace one another. When both compounds were absent from the basal medium no continued growth took place. Both stimulated the growth of cultures (*Lactobacillus manni* and *Lactobacillus lyophilophilus*), but neither compound was essential in the presence of the other. This is very interesting from the point of view of essential metabolism, since it suggests either that the organisms can use alternative metabolic reactions, according to whether riboflavin or thiamin is available; or possibly the presence of the one substance allows the synthesis of the other. In any case the observation throws interesting light on the conception of essential metabolism and suggests that here too the 'essential' may be only relatively so.

The part of thiamin in the nutrition of propionic acid bacteria was further illuminated by Wood, Andersen, and Werkman (435) and by Silverman and Werkman (346) who found it possible to train certain strains, which originally required the addition of thiamin, to dispense with this substance and to give vigorous and continued growth on a basal medium not containing it. Serial transfer in a medium deficient in thiamin was the method

used, and it was shown (347) that thiamin was synthesized by the trained organisms (*Propionibacterium pentosaceum*) which could phosphorylate the compound to co-carboxylase.

In the case of *Staph. aureus* no evidence of such training to synthesize thiamin has been recorded. Hills (127), in his study of pyruvate metabolism by *Staph. aureus*, grew his organisms in minimal concentrations of thiamin and was thus able to obtain thiamin-deficient cells which showed sharp responses to the addition of thiamin in metabolism experiments. If the organisms he used had shown a tendency to synthesize thiamin under these

TABLE II  
Utilization of Thiamin in Bacterial Growth

Organism	Thiamin				References
	is essential nutrient	acceler- ates growth	is not essen- tial as added nutrient	is synthe- sized	
<i>Staphylococcus aureus</i>	+	—	—	—	155, 156, 288
“ <i>albus</i>	+	—	—	—	350
“ <i>flavus</i>	—	—	—	(+)	200
<i>Streptococcus salivarius</i>	+	—	—	—	272a
<i>Brucella</i> :					
Some strains of <i>B. suis</i>	+	—	—	—	166
“ “ “ <i>B. melitensis</i>	+	—	—	—	
“ “ “ <i>B. abortus</i>	+	—	—	—	
<i>Salmonella gallinarum</i>	+	—	—	—	141
<i>Bacillus larvae</i>	+	—	—	—	206
Propionic acid bacteria:					
a	+	—	—	—	346, 381, 436
b	—	+	—	+	
c	—	—	+	+	
Lactic acid bacteria, various strains	—	+	?	(+)	249, 251, 437
<i>Serratia marcescens</i> , <i>Pseudomonas aeruginosa</i> , <i>B. subtilis</i> , <i>Eberthella typhosa</i>	—	—	—	+	156
Various dysentery bacilli	—	—	—	+	66
<i>C. diphtheriae</i>	—	—	—	+	83

conditions (which were favorable for such a process), less clear-cut results would have been expected.

Other bacteria whose relation to thiamin has been studied are listed in Table II.

There are a few indications as to the way in which thiamin is used by bacteria. In *Staphylococcus aureus*, Hills (127) showed that the anaerobic dismutation of pyruvate to lactate, acetate, and CO<sub>2</sub> depended on the presence of thiamin. Smyth (350), using strains of *Staph. aureus* and *Staph. albus*, found that oxaloacetate could replace thiamin for limited periods in

this reaction. Apparently the function of the thiamin was to catalyze the formation of oxaloacetate from pyruvate and  $\text{CO}_2$ , the oxaloacetate then acting as a hydrogen carrier in the formation of lactate and acetate from pyruvate. Provision of oxaloacetate could therefore temporarily remove the need for added thiamin, while added oxaloacetate remained; thiamin was required for the continued production of oxaloacetate, as in growth. Quastel and Webley (289) showed that acetic acid oxidation by certain "propionic acid" (270) bacteria required thiamin.

### 2. Adaptation in Thiamin Synthesis by Bacteria

In the groups of lactic acid and propionic acid bacteria the presence of thiamin in the nutrient medium is not under all conditions 'essential', but its effect in stimulating the rate of growth can be shown. This must be considered in the light of "training" experiments (346, 347, 435, 436). These show that, not only is the demand for thiamin by various cultures subject to change, but the need for pre-formed thiamin in the nutrient medium is relative to the organism's ability to synthesize the substance. The dispensability of thiamin as a nutrient is relative to the ability of the organism to synthesize it, and does not mean that it is no longer required in the metabolism of the organism.

### 3. Bacterial Synthesis

To complete the picture we must include the numerous other species which have been directly shown to synthesize thiamin. These include all the bacteria examined which have been chosen at random from among those able to grow on simple media not containing thiamin, for example: *Pseudomonas aeruginosa*, *B. subtilis*, *Eberthella typhosa*, *Serratia marcescens* (156), *C. diphtheriae* (83), *Rhizobium trifolii* (408, 409, 410); some dysentery bacteria (66), *Phytomonas tumefaciens* (244). With *Rhizobium trifolii* and *Phytomonas tumefaciens* the rate of synthesis was slow enough for an acceleration of growth to be observed when thiamin was added. When a stimulation of growth can be shown it is logical to conclude that the added substance plays an important part in the metabolism of the organism, especially when this is coupled with the fact of synthesis by the organism. In the case of those organisms which synthesize thiamin but for which no acceleration of growth on adding thiamin has been recorded (perhaps because it has not been specially sought, since the organisms grew normally on ordinary media) it may be assumed that the rate of synthesis is great enough for it not to be a limiting factor under the conditions of test. It also seems safe to assume that the thiamin synthesized by bacteria is used in essential metabolic reactions, and that the synthesis is not a vestigial process no longer of use to them. On the contrary, there is much cumulative evidence

for the view that the metabolic reaction using thiamin is very general in the physiology of bacteria and many other types of organism. The place of thiamin in nutrition is best viewed as a special aspect of its place in metabolism, namely that it appears as a nutritional requirement when it cannot be synthesized adequately.

West (407) made a very interesting observation on the nutrient requirements of the bacteria in the soil of the rhizosphere of growing plants, as compared with the requirements of bacteria elsewhere in the soil and distant from the rhizosphere. The nutrient requirements were examined by using one medium not containing growth factors and a similar medium with added growth factors (thiamin,  $\beta$ -alanine, nicotinic acid, inositol, biotin). Of 100 species isolated from the rhizosphere a majority required growth factors, while of 100 species isolated from soil away from the rhizosphere the majority did not require these essential metabolites as nutrients. The suggestion was that the former group, of more exacting nutritional requirements, derived the substances they needed from material excreted by the roots. West showed that thiamin (assayed by *Staph. aureus*) and biotin (assayed by *Rhizobium trifolii*) were excreted by flax-seed grown in a sterile medium. The existence of essential metabolites in the rhizosphere due to excretion from the roots would favor the selection in that sphere of a population of micro-organisms more or less dependent on an external source of essential metabolites. Elsewhere in the soil, the lack of these essential metabolites would favor the selection of micro-organisms with greater synthetic powers. A relation of this type may have influenced the evolution of the nitrogen-fixing legume nodule bacteria from free-living forms (see p. 165).

The place of thiamin in bacterial physiology is summarized in Table II. This should be read only in the light of what is written above, since the rigidity of categories necessarily given by a table suggests an unreal simplification. Since the synthetic abilities of micro-organisms can alter, and can be changed deliberately, and the rate of synthesis may determine nutritional needs, the classifications are conditioned by this biological flexibility.

#### 4. *Thiamin in the Growth of Organisms Other than Bacteria*

The picture of the ways in which thiamin is related to bacterial growth is paralleled with other types of organism. In particular, molds, lower fungi, protozoa (118) and the isolated roots of higher plants have been extensively examined. A collection of the observations illustrating the parallelism with the bacterial findings is given in Tables III, IV, and V.

#### 5. *Use of the Pyrimidine and Thiazole Portions of Thiamin*

It was found for *Staphylococcus aureus* that thiamin could be replaced by the component pyrimidine and thiazole portions of the molecule given to-



gether (156). This observation was extended to the lower fungi and protozoa and isolated roots of higher plants. It became clear that there was the same general trend in the relative degree of importance of thiamin in the nutrition of these organisms, and this was shown in rather more detail than with bacteria. It was found that some organisms required the intact thiamin molecule, others could use the separated pyrimidine and thiazole portions, while still other species needed to be given *only* the pyrimidine or the thia-

TABLE III

*A Selection of Lower Fungi Showing Relative Nutritional Requirements for Thiamin or Its Components*

Organism	Minimal nutrient requirements:			References (General: 309)
	Thiamin	Pyrimidine	Thiazole	
<i>Phytophthora boehmeriae</i>	+	—	—	
<i>P. cactorum</i> , <i>P. cinnamomi</i>				
etc. (about 10 species)				
<i>Trichophyton discoides</i>	+	—	—	318
<i>Ceratostomellae: fimbriata</i> , London	+	—	—	317
plane, <i>penicillata</i>				
<i>Chalaropsis thielavioides</i>				
<i>Phycomyces blakesleeianus</i>	—	+	+	38, 303, 333, 337, 348
<i>Phycomyces nitens</i>	—	+	+	198, 307
<i>Ceratostomellae: ips</i> , <i>pini</i> , <i>radici-</i>	—	+	+	317
<i>cola</i> ; <i>Polyporus versicolor</i>				
<i>Phytophthora fagopyri</i>				
<i>Sclerotium delphina</i> , <i>Rolfsii</i>	—	+	—	307
<i>Sphaerulina trifolii</i>	—	+	—	307
<i>Pythium polycladon</i>	—	+	—	307
<i>Pythium butleri</i>	—	+	—	307, 308
<i>Melanospora destruens</i> formation	—	+	—	125
of perithecia				
<i>Ceratostomellae: microspora</i> , <i>mon-</i>				
<i>tium</i> , <i>obscura</i> , <i>piceaperda</i> , <i>steno-</i>	—	+	—	317
<i>cerus</i>				
<i>Mucor ramannianus</i>	—	—	+	144, 269, 317, 332
<i>Absidia glauca</i> and numerous	—	—	—	307
other lower fungi				

zole part, in which case the complementary component of the thiamin molecule was found to be synthesized. This is illustrated in Tables III, IV, and V.

It may be concluded that all the organisms need thiamin for the performance of some common metabolic reactions. This generalization is further supported by the examination of the biological specificity of the thiamin molecule (see section 8) which is found to be high, and, except for minor differences, to be the same for bacteria, fungi, protozoa, and isolated roots.

In addition to the organisms mentioned in Tables II-V, it may be mentioned that Robbins and Kavanagh (304) examined 8 species of *Torula* and found, as with other organisms, that some species required an external supply of thiamin or its pyrimidine and thiazole components together, some required the pyrimidine and synthesized the thiazole, and some synthesized

TABLE IV

*The Requirements of Various Species of Ustilago for Thiamin or Its Components (26a, 336)*

Species	Required as nutrient:			Synthesized:		
	Thiamin	Pyrimidine	Thiazole	Thiamin	Pyrimi-	Thiazole
<i>U. scabiosae</i>	+	or	+	-	-	-
<i>U. violacea</i> (+ and -)	+	±	+	-	slowly	slowly
<i>U. violacea</i> f. <i>sp. melandrya</i>						
<i>U. longissima</i> (+ and -)						
<i>U. zeae, tritici, levis</i>	-	-	-	+	+	+
<i>nuda, hordei,</i>						
<i>avenae, bromivora</i>						

TABLE V

*Requirements of Various Protozoa for Thiamin or Its Components\**

Organism	Required as nutrient:			References
	Thiamin	Pyrimidine	Thiazole	
<i>Glaucoma piriformis</i>	+	-	-	79, 223
<i>Strigomonas oncopelti</i>	+	-	-	225
“ <i>fasciculata</i>	+	-	-	226
“ <i>culicidarum</i>	+	-	-	226
<i>Euglena pisciformis</i>	-	+	+	73
<i>Chilomonas paramecium</i>	-	-	+	214, 216
“ <i>oblonga</i>	+	?	?	274
“ <i>oblonga longata</i>	+	?	?	
“ <i>globosa</i>	+	?	?	
“ <i>coniformis</i>	+	?	?	
<i>Polytomella caeca</i>	-	+	+	213, 216, 274
“ <i>agilis</i>	+	?	?	274
“ <i>globosa</i>	-	?	?	274
<i>Polytoma ocellatum</i>	-	-	+	215, 216
“ <i>caudatum</i>	-	-	+	214, 216, 219
“ <i>obtusum</i>	-	-	-	216
“ <i>uvella</i>	-	-	-	216

\* For specificity see (217, 218); cf. also addl. ref. (210b, 226a).

both components. Schopfer (333) found that *Rhodotorula flava* and *Rt. rubra* required only the pyrimidine and synthesized the thiazole.

### 6. Yeasts

Although yeasts were among the earliest micro-organisms for which effects on growth by thiamin preparations were shown (429), the substance

appears to be synthesized more or less rapidly by most yeasts. There is apparently no case of a yeast for which thiamin is an indispensable nutrient. Even "old process" yeast can grow continuously in the absence of added thiamin, although very responsive to the addition of thiamin under certain conditions (425). The effects of thiamin and its components on yeast growth and fermentation have been studied by Schultz, Atkin, and Frey (338, 339, 341).

### 7. Thiamin in The Growth of Isolated Roots of Higher Plants

White (416, 417) cultivated isolated tips from tomato roots in continuous passage for several years under sterile conditions *in vitro*, using a medium containing sucrose, nitrate, inorganic salts, and 0.01% yeast extract. The yeast extract was essential. It was found that thiamin was the substance of primary importance supplied by yeast extract under such conditions for pea-roots (29) and for tomato-roots (311, 417). The need for thiamin in the growth of isolated roots of numerous higher plants has now been extensively studied (2, 30, 33, 37, 38, 39, 61, 312, 313, 314). Bonner and Greene (41, 42), using the *Phycomyces* assay to determine the distribution of thiamin, showed directly that thiamin was photosynthesized in the leaves and transported to the roots. For slow growing plants, the rate of growth was accelerated by the addition of thiamin to the root nutrients; this did not occur with fast growers, where the rate of thiamin synthesis and transport was evidently not a limiting condition.

As with the other organisms already discussed, the effect of the two components of thiamin on root growth has been examined. Robbins and Schmidt (312) found that the vitamin thiazole component alone had considerable effect, while the pyrimidine component alone was ineffective. Some synthesis of pyrimidine in the root was indicated by *Phycomyces* assay. Thiazole and pyrimidine together were as effective as thiamin itself (30, 312).

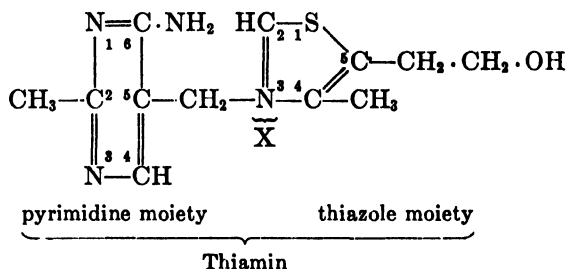
### 8. Specificity

When it was found that the pyrimidine and thiazole components of thiamin could often replace thiamin, the opportunity was taken to examine the question of the biological specificity of the molecule, since a large number of related pyrimidines and thiazoles had been prepared by the workers engaged in the synthesis of thiamin. Detailed studies on the specificity of the pyrimidine and thiazole portions of thiamin and of thiamin analogs have been made for *Staph. aureus* (159); *Phycomyces blakesleeana* (38, 305, 306, 333, 334, 348); for various protozoa (236, 237) and for pea-roots (29, 30, 37). [Comparative summaries are given in 159, 211, 309].

The structure of thiamin (or of its component rings) was found to be very specific; few alterations could be made without seriously affecting the bio-

logical activity. This high degree of biological specificity was common among all the organisms examined.

Rather than detail the results with the numerous compounds examined it will be sufficient to note some of the few points of difference in availability which have been observed as between different organisms. These differences are all relatively minor and are most easily viewed as indications either of the ability of the various organisms to convert a related compound to the master compound or, in some cases, possibly of a functional use of the derivative.



a) *Pyrimidine Ring Substitution.* The effect of varying the substituents in the pyrimidine ring has been tested in the presence of equimolar or greater concentrations of the vitamin thiazole. For *Staph. aureus* the results of altering the substitution in the pyrimidine ring show that essential groups required in that ring are: a  $-\text{CH}_3$  group at 2 and an  $-\text{NH}_2$  group at 6. This holds also for the protozoa examined by Lwoff and Dusi (216, 217, 218, 219), and for *Phycomyces blakesleeanus* and pea-roots (30). A thiamin analogue ('isothiamin') having  $\text{CH}_3$  at 4, instead of 2, and a 'chlorothiamin' having  $\text{Cl}$  at 2 and  $\text{CH}_3$  at 4, both had about  $1/10,000$  of the activity of thiamin for *Staph. aureus*; the 'chlorothiamin' was inactive (at  $10^{-7}$ ) for isolated pea-roots (30).

When an equimolar mixture of pyrimidine and thiazole was given to *Staph. aureus* instead of thiamin the nature of the substitution at 5 in the pyrimidine was important. Thus,  $-\text{CH}_2 \cdot \text{NH}_2$ ,  $-\text{CH}_2 \cdot \text{OH}$  and  $-\text{CH}_2 \cdot \text{NH} \cdot \text{CSH}$  permitted growth but  $-\text{CH}_2 \cdot \text{CO} \cdot \text{NH}_2$  or  $-\text{CH}_3$  did not. The relative effects of these various substituents at position 5 was interpreted (159) to mean that for use the pyrimidine and thiazole portions were united by *Staph. aureus* to give the thiazolium compound thiamin itself, and that the two components were not used separately. With pea-roots similar differences are found in the availability of the substitution at 5; these also appear to be related to the ability of the root-cells to form the link with the thiazole. Thus  $-\text{CH}_2\text{Br}$  (100%),  $-\text{CH}_2 \cdot \text{NH} \cdot \text{CSH}$  (100%)  $-\text{CH}_2 \cdot \text{NH}_2$  (95%) and  $-\text{CH}_2 \cdot \text{OEt}$  (25%) decrease in availability in the order shown, while  $-\text{H}$ ,  $-\text{CH}_2 \cdot \text{COOH}$  and  $-\text{CH}_2 \cdot \text{CO} \cdot \text{NH}_2$  could not be used (30).

The substituted  $-\text{CH}_2-$  groups at 5 in the pyrimidine which were usable were the same for the four flagellate leucophytes examined by Lwoff and Dusi (217, 218, 219) as for *Phycomyces* and pea-roots. Thus all observations coincide in expressing a similar structural specificity for all the organisms examined, both for groups in the ring and in the group through which the linkage to the thiazole would be made to form thiamin.

Direct evidence for the synthesis of thiamin by joining the pyrimidine and thiazole components was shown for *Phycomyces blakesleeanus* by Bonner and Buchman (38). They further showed that after growth the resting mycelium broke down the thiamin, with destruction of the thiazole and liberation of the free pyrimidine. With *Staph. aureus* Hills (127) found similarly that the destruction of the thiazole was more rapid than that of the pyrimidine.

Those organisms (e.g., *Glaucoma piriformis* and *Phytophthora cinnamomi*) which require the intact thiamin molecule and cannot use the two components, evidently cannot link them together as other protozoa, *Staph. aureus*, *Phycomyces blakesleeanus*, and pea-roots can when the substitution in the 5  $-\text{CH}_2$ -group is suitable.

b) *Thiazole Ring Substitution*. The structural specificity of the thiazole portion of thiamin (tested in the presence of equimolar or greater concentrations of the vitamin pyrimidine) is less strict than it is for the pyrimidine component. If the 5- $\beta$ -hydroxyethyl group proper to the thiamin thiazole be replaced by a 5- $\gamma$ -hydroxypropyl or a 5- $\beta$ -hydroxypropyl group there is a much decreased biological activity, both with *Staph. aureus* and the four flagellate leucophytes of Lwoff and Dusi. Similarly with *Phycomyces blakesleeanus* only 1% or less of the growth given by thiamin was obtained (40, 306). The presence of a  $\beta$ -alanyl side chain instead of the 5- $\beta$ -hydroxyethyl group rendered the thiazole inactive for *Staph. aureus* and *Phycomyces* (47, 48, 120) but it was used by the flagellate leucophytes examined by Lwoff and Dusi, and by pea-roots (40). The flagellate leucophytes could also use a thiazole with a  $\beta$ -aminoethyl group at 5, unlike *Staph. aureus*. A  $\text{CH}_3$  group at 2 rendered the thiazole inactive for *Staph. aureus* and for *Phycomyces*, lowered the activity to 30% for pea-roots (30) but it was active for the flagellate leucophytes *Polytoma caudatum*, *P. ocellatum* and *Polytomella caeca* (218). The corresponding 2-hydroxy-4-methyl-5- $\beta$ -acetoxyethyl-thiazole was inactive for the leucophytes and for *Staph. aureus*. Thiamin analogs having 5- $\beta$ -hydroxypropyl and 5- $\gamma$ -hydroxypropyl groups in the thiazole ring were used by *Glaucoma piriformis* and *Strigomonas oncopelti*, but were much less potent than thiamin itself (224, 226).

With pea-roots, it was found (30) that for a positive effect an-OH group or a group metabolizable to-OH was required. But there was a relatively wide range of positions which this could occupy (see Table VI) and still

permit growth. Compounds 2, 3, and 4 show that the effect of moving the OH along a side chain at position 5 in the thiazole ring is not great; and the effectiveness of compounds 5, 6, and 7 is probably related to their hydrolysis to the corresponding hydroxycompounds by the root cells. Compounds 8 and 9 show that the side-chain bearing the —OH group can be altered to the 4-position without making the derivative useless.

The lowered activity (35%) of compound 10, where a methyl group is substituted for the H at position 2 in the vitamin thiazole, and the complete inactivity of compound 11, where there is —NH<sub>2</sub> instead of —H at the same position, is very interesting, and indicates that the 2 position can have some effect in determining the metabolic use of the thiazole, since these compounds both contain the 5-CH<sub>2</sub>·CH<sub>2</sub>·OH group of the vitamin thia-

TABLE VI

*The Activity of Thiazoles (at 10<sup>-7</sup> M) in the Growth of Isolated Pea-Roots, [the Thiamin Pyrimidine Being Present in Excess] (30)*

Compound	Substituents in thiazole ring in positions indicated:			Relative efficiency (% of thiamin thiazole activity)
	position 5	position 4	position 2	
1.	—CH <sub>2</sub> ·CH <sub>2</sub> ·OH*	CH <sub>3</sub>	H	100
2.	—CH(OH)·CH <sub>3</sub>	CH <sub>3</sub>	H	30
3.	—CH <sub>2</sub> ·CH(OH)·CH <sub>3</sub>	CH <sub>3</sub>	H	100
4.	—CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·OH	CH <sub>3</sub>	H	75
5.	—CH <sub>2</sub> ·CH <sub>2</sub> Cl	CH <sub>3</sub>	H	100
6.	—CH <sub>2</sub> Br	CH <sub>3</sub>	H	90
7.	—CH:CH <sub>2</sub>	CH <sub>3</sub>	H	100
8.	—CH <sub>2</sub> ·CH <sub>3</sub>	CH <sub>2</sub> ·OH	H	100
9.	—CH <sub>3</sub>	CH <sub>2</sub> ·CH <sub>2</sub> Cl	H	75
10.	—CH <sub>2</sub> ·CH <sub>2</sub> ·OH	CH <sub>3</sub>	CH <sub>3</sub>	35
11.	—CH <sub>2</sub> ·CH <sub>2</sub> ·OH	CH <sub>3</sub>	NH <sub>2</sub>	0

Inactive were: —CH<sub>2</sub>·CH<sub>3</sub>, —CO·CH<sub>3</sub>, —CH<sub>2</sub>·COOH, —CHO, —H at 5, each with the correct CH<sub>3</sub> at 3 and H at 2.

zole. It would be interesting to know whether compound XI has any inhibitory effects on the activity of the vitamin thiazole (30).

### 9. Biogenesis of Thiamin

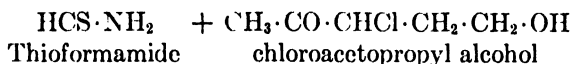
Buchman and Richardson (47) and Harington and Moggridge (120) independently examined the possibility that the thiazole portion of thiamin might be derived from a precursor  $\alpha$ -amino acid compound by a reaction similar to that carried out by fermenting yeast on the amino acids phenylalanine, tyrosine, and tryptophan, which yield the corresponding  $\beta$ -phenylethyl alcohol, tyrosol and tryptophol, respectively (78). They saw that the —CH<sub>2</sub>·CH<sub>2</sub>·OH group might be derived from a similar reaction with an  $\alpha$ -aminopropionic acid (alanine) precursor. The compound  $\alpha$ -amino- $\beta$ -(4-

methylthiazolyl-5)-propionic acid was synthesized. Neither *Staph. aureus* nor *Phycomyces blakesleeanus* could utilize the compound, but pea-roots were able to do so (37).

Information about biosynthesis of thiamin in pea-roots was also obtained from the examination of specificity and illustrates the very useful weapon that such studies with any essential metabolite may give.

Bonner and Buchman (37) used the *Phycomyces* assay to determine the fate of those thiazole derivatives which supported the growth of pea-roots. Derivatives with the following groups at position 5:  $-\text{CH}_2 \cdot \text{CH}_2\text{Cl}$ ,  $-\text{CH} : \text{CH}_2$ ,  $-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{SH}$ ,  $-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH}_2$ ,  $-\text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$ , all of which supported growth, were all found to yield the vitamin thiazole, showing that the root-cells could convert these chains to  $-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$ . But the derivatives with  $-\text{CH}(\text{OH}) \cdot \text{CH}_3$  (II),  $-\text{CH}_2 \cdot \text{CH}(\text{OH}) \cdot \text{CH}_3$  (III) at 5, and compound X (with  $-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$  at 5 but with  $-\text{CH}_3$  at 2), although able to support growth, were not converted to the vitamin thiazole; that is, a functional use was made of these compounds as such. This brings out clearly that biological specificity in an essential metabolic process may be affected in at least two ways; (a) by conversion to the master-compound, (b) by functional use of a derivative, both conditioned by the biological adaptability of the organism.

Some information about biosynthesis in pea-root cells was gained also by an examination of the use of possible precursors in thiazole synthesis. Thus it was found that the reaction:



which gives the vitamin thiazole *in vitro*, was also carried out by the root-cells. The latter could moreover carry out the condensation using acetopropylalcohol itself, which does not occur *in vitro*. But the root-cells could not condense  $\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}(\text{OH}) \cdot \text{CH}_3$  with thioformamide to give 4-methyl-5- $\alpha$ -hydroxyethyl-thiazole (compound II, Table VI). This compound has some activity (30%) in root growth, however. The roots, which can make a functional use of it, cannot synthesize it from appropriate precursors as they can the vitamin thiazole. There is thus a considerable specificity in the synthesizing enzyme systems of the root-cells; there is apparently a 'thiaminase' which can link appropriate pyrimidines and thiazoles; and a 'thiazolase' which can effect ring-closure to thiazole from suitable precursors. In their discussion of the degree of specificity of different thiazoles in the growth of *Phycomyces*, Bonner and Erickson (40) suggested two reasons which might account for the fact that a number of thiazoles, differing somewhat from the thiamin thiazole, nevertheless had considerable biological activity. They suggested that the carboxylase proteins of

different organisms might differ so that slightly different thiamin analogs gave the most active enzyme system in particular organisms. Secondly they suggested that thiamin might have more than one physiological function. Different physiological functions might demand different structural requirements. In any one organism the physiological function of greatest importance would determine the structural specificity. Thiamin itself would be the integrated structure possessing the optimum configuration for all the physiological activities in which it took part. Thiamin pyrophosphate is co-carboxylase in the enzymic decarboxylations of pyruvate, and this must be one of the functions for which thiamin is required.

#### 10. Specific Inhibition of Thiamin Utilization

Buchman, Heegard, and Bonner (48) observed a very interesting inhibition of the activity of a carboxylase system by the pyrophosphate of the vitamin thiazole (4-methyl-5- $\beta$ -hydroxyethyl-thiazole pyrophosphate). This inhibition was explained as due to a competition between co-carboxylase (thiamin pyrophosphate) and the thiazole pyrophosphate, for the carboxylase protein, the thiazole pyrophosphate giving an inactive "enzyme analogue." On the other hand, Niven and Smiley (272a) found that co-carboxylase was 40% more active than intact thiamin for *Streptococcus salivarius*. (See also Sarett and Cheldelin, addl. ref., 325a).

In more detail the theory of specific anti-metabolite growth inhibitions is well illustrated by the behavior of the thiamin analogue pyrithiamin, which is 1-[(4-amino-2-methyl)-5-pyrimidylmethyl]-2-methyl-3-( $\beta$ -hydroxyethyl) pyridinium bromide hydrobromide, *i.e.*, it is a thiamin analogue in which a pyridine ring takes the place of the thiazole ring of thiamin. Pyrithiamin was tested as a substitute for thiamin by Robbins (301) using three fungi which required thiamin or portions of the molecule, for growth. *Phycomyces blakesleeanus*, which used thiamin or equimolar mixtures of the vitamin thiazole + pyrimidine, was unable to use pyrithiamin, but it split the molecule and used the pyrimidine portion, if supplied with the vitamin thiazole; the pyridine analogue of the thiazole was useless. *Phytophthora cinnamomi*, which requires the intact thiamin molecule and cannot utilize a vitamin pyrimidine + thiazole mixture, also could not use pyrithiamin. *Pythiomorpha gonapodioides* synthesizes the thiamin thiazole and requires only to be given the thiamin pyrimidine. This organism could use pyrithiamin as source of the thiamin pyrimidine at a level of 0.5 to 1.0  $m\mu$  mol./25 ml. of basal medium, but at levels above 2  $m\mu$  mol. the compound was toxic. Some evidence of toxicity was also noticed with the other two fungi but a quantitative study of its toxicity *vis d vis* thiamin was not made. Clearly enough, pyrithiamin was not an active isostere of thiamin, and its activity at low concentrations was due only to its being a source of the thiamin pyrimidine for those organisms which could not synthesize this.



The specific anti-thiamin inhibitory effect of pyrithiamin was clearly shown by Woolley and White (453) who tested several different species of bacteria, yeasts, and fungi for ability to grow in presence of pyrithiamin. Without exception, those organisms which needed thiamin in the medium for maximal growth, *i.e.*, which required pre-formed thiamin and could not synthesize it, were all prevented from growth by small concentrations of pyrithiamin. On the other hand, those organisms which did not require

TABLE VII

*Inhibitory Power of Pyrithiamin for Various Micro-organisms Correlated with Requirement of Thiamin as Growth Factor (453)*

Inhibition index:  $\mu\text{g.}$  pyrithiamin required to reduce to  $\frac{1}{2}$  maximal, growth promoted by  $0.01 \mu\text{g.}$  thiamin/ml. medium, in case of those organisms which require thiamin. For those organisms which did not require added thiamin, the media contained none added and could not have contained  $> 0.001 \mu\text{g./ml.}$  (by assay). This figure was taken in calculating the inhibition index.

Organism	Inhibition index pyrithiamin/thiamin	Thiamin requirement of the organism
<i>Ceratostomella fimbriata</i>	7	Intact thiamin
“ London plane	19	“ “
“ <i>penicillata</i>	10	“ “
<i>Phytophthora cinnamomi</i>	12	“ “
<i>Chalaropsis thielavioides</i>	11	“ “
<i>Endomyces vernalis</i>	130	pyrimidine
<i>Mucor ramannianus</i>	800	thiazole
<i>Saccharomyces cerevisiae</i>	800	pyrimidine + thiazole
<i>Salmonella gallinarum</i>	1000	“ “
<i>Staphylococcus aureus</i>	2000	“ “
<i>Neurospora crassa</i>	400,000	None
<i>Escherichia coli</i>	2,000,000	“
<i>Clostridium butylicum</i>	2,000,000	“
<i>Lactobacillus arabinosus</i>	40,000	“
“ <i>casei</i>	5,000,000	“
“ <i>delbrückii</i>	5,000,000	“
“ <i>mesenteroides</i>	5,000,000	“
“ <i>pentoaceticus</i>	5,000,000	“
<i>Streptococcus lactis</i> R	5,000,000	“
<i>Propionibacterium pentosaceum</i>	5,000,000	“
<i>Streptococcus hemolyticus</i> H 69 D	4,000,000	“

thiamin to be given (and which synthesized it) were not inhibited even by considerable concentrations of pyrithiamin.

Table VII shows the inhibitory power of pyrithiamin for various micro-organisms, correlating this with the requirement of thiamin as a nutrient. In each case of inhibition the effect of pyrithiamin could be abolished by a suitable concentration of thiamin; with *Endomyces vernalis*, which requires only the thiamin pyrimidine for growth, this portion of thiamin alone could

abolish the pyrithiamin inhibition. But *Endomyces vernalis* and *Mucor ramannianus*, which both synthesized thiamin when given, respectively, the correct pyrimidine or thiazole, apparently did not synthesize pyrithiamin when given the pyridine analogue 2-methyl-3-hydroxyethyl-pyridine, since neither was inhibited by the pyridine analogue up to a concentration of 100  $\mu\text{g./ml.}$ , which could have produced an inhibitory concentration of pyrithiamin. By using the growth response of *Endomyces vernalis* the synthesis of thiamin by those organisms which did not require added thiamin (or thiamin component) was measured quantitatively. The amount of thiamin synthesized, though clearly adequate for the growth of the organisms, did not seem to be enough to antagonize the quantities of pyrithiamin used in the inhibition tests. With *Esch. coli* the presence of pyrithiamin did not induce increased production of thiamin, nor were antagonists to pyrithiamin synthesized (other than the mainly intracellular thiamin). Thus the resistance of these organisms was not due to the amounts of thiamin they synthesized, though sufficient quantities of thiamin could reverse pyrithiamin inhibition.

To study the mode of inhibition by pyrithiamin, Woolley (447) developed a pyrithiamin-resistant strain (PF = pyrithiamin-fast) of *Endomyces vernalis* by sub-cultivation in the presence of pyrithiamin; the resistant strain grew as well in presence of 23  $\mu\text{g.}$  pyrithiamin/ml. as it did in the absence of the compound. This concentration was 25 times that sufficient to inhibit half maximally the parent strain. The PF strain grew somewhat more slowly than the parent strain; its thiamin requirement remained the same, either thiamin or the thiamine pyrimidine being required for growth. Thus the trained PF strain had not become able to synthesize thiamin, as has been observed with training lactic and propionic acid bacteria to dispense with other essential nutrients including thiamin (346, 347, 435, 436). In the absence of added thiamin, the pyrithiamin was itself a growth factor for the PF strain. Several organisms were found to grow slightly better in some sub-inhibitory concentrations of pyrithiamin than in its complete absence. All these organisms were thiamin-synthesizers, with the exception of *Mucor ramannianus* which synthesizes only the thiamin pyrimidine. These growth stimulations by sub-inhibitory concentrations of pyrithiamin may have been due to the substance supplying easily available thiamin pyrimidine, the inhibition effect not operating at the low concentrations used, as shown by the direct test (see Table VII). The resistance of the PF strain of *Endomyces vernalis* to pyrithiamin was partly explained by the observation that pyrithiamin was destroyed and the pyrimidine portion of the molecule (identical with that in thiamin) was released during growth in presence of pyrithiamin. Thus the PF strain had developed a pyrithiamin-splitting system which the parent strain did not possess. The parent strain could grow

when given only the thiamin pyrimidine, and the splitting of pyrithiamin by the trained PF strain would explain why pyrithiamin could be a growth factor for that strain, namely because it acted as a source of the vitamin pyrimidine. Destruction of pyrithiamin by the PF strain was, however, not the only means by which the PF strain had become resistant, because more than enough pyrithiamin to cause inhibition of the parent strain could be demonstrated in the culture filtrate in which the PF strain had been grown; this inhibition could be abolished by thiamin, and was not due to the production of other antagonists. Other reasons for the resistance still remain obscure. Clearly pyrithiamin has marked selective inhibitory properties: organisms which cannot synthesize thiamin are strongly inhibited, and the more marked the need for thiamin the easier is the inhibition, while the thiamin synthesizers are not inhibited. The inhibition appears to be directly connected with thiamin function in the non-synthesizers of thiamin. With mice, a thiamin-deficiency disease could be produced by feeding pyrithiamin, which was cured by thiamin (454). But the failure of thiamin-synthesizers to be inhibited is not directly connected with their ability to synthesize sufficiently large amounts of thiamin. The ability to split pyrithiamin also does not account for the resistance of the thiamin-synthesizers, though it may account for the growth stimulation of sub-inhibitory concentrations. It looks as if pyrithiamin can block the intake of thiamin which is essential for the growth of the non-synthesizers, but pyrithiamin is unable to make effective adverse intrusion into the metabolic chain when thiamin is synthesized intra-cellularly.

The exact mode of action of pyrithiamin is thus still obscure; what is clear is that it must be closely concerned with interference with thiamin function, and this inhibition is due to *structural similarity* with the essential metabolite with which it interferes.

### III. RIBOFLAVIN

#### 1. General Considerations

Riboflavin (vitamin B<sub>2</sub>): 6,7-dimethyl-9-(*d*-1-ribityl)-isoalloxazine is known to be an integral part of several important enzyme systems of wide distribution in biological materials of all kinds (yeast, mammalian tissues, milk). It occurs in the prosthetic group of these enzymes, which are concerned with the mechanisms of cellular oxidation (108), particularly in the metabolism of various organisms of the lactic and propionic acid producing groups, of hemolytic streptococci and of some luminescent bacteria. The synthesis of riboflavin by various bacteria has also been recorded. Riboflavin is an important nutrient for some strains of lactic and propionic acid bacteria; other strains are stimulated by it, while still other strains synthesize riboflavin and no stimulation of growth is seen on adding it. Riboflavin

thus appears to be involved in bacterial growth in the same relative way already described for thiamin. There are metabolic mechanisms, important in the physiology of widely different species, in which riboflavin has an integral part, and the dependence of any particular species or strain of bacteria on an external source of supply of riboflavin is relative to the ability of the given organism to synthesize it. Alterations in response to the addition of riboflavin during subculture have been studied. The observations concerning riboflavin and bacterial growth are summarized in Table VIII.

TABLE VIII

*Riboflavin in the Metabolism of Various Bacteria*

Organism	Riboflavin effect
Hemolytic streptococci of various Lancefield groups	Essential nutrient (448, 449)
<i>Strept. fecalis</i>	Essential nutrient (342)
Lactic acid bacteria	Growth response relative to ability to synthesize; this is not fixed but can change. Thus for some strains riboflavin is a necessary nutrient, for others a stimulant, and for others no stimulation is observed (157, 362, 363, 437)
Propionic acid bacteria	Growth stimulation; strains can be trained to dispense with its addition, probably by becoming able to synthesize it (436)
Certain luminescent bacteria	Riboflavin involved in growth and luminescence; some variants lose ability to synthesize and then respond to addition of riboflavin (69)
Dysentery bacilli	Riboflavin synthesized; not required as nutrient (66)
<i>Staph. aureus</i>	Riboflavin synthesized; not required as nutrient (273)
<i>Rhizobium trifolii</i>	Riboflavin synthesized; may be required in initiating growth (409)
<i>C. diphtheriae</i>	Riboflavin synthesized (83)

*2. Streptococci*

Recent studies of the exact nutritional requirements of various strains of hemolytic streptococci have resulted in their cultivation on media of completely defined chemical composition (232, 342, 449). Riboflavin has been included in the media in all cases, on the grounds of its known behavior as a growth factor for other bacteria, but it has not been shown always to be an essential component of the media. For the strains of hemolytic streptococci grown by Woolley and Hutchings (449) and by Schuman and Farrell (342), which included members of Lancefield's groups A, B, C, D, riboflavin was essential; also for *Streptococcus fecalis* (342). Earlier work, before these media of defined composition had been worked out, had indicated the important part played by riboflavin in the nutrition of various strains of hemolytic streptococci (290, 448).

### 3. *Lactic Acid Bacteria*

Riboflavin was first shown to be an important nutrient for bacteria by Orla-Jensen, Otte, and Snog-Kjaer (275) who worked with a number of lactic acid bacteria. Numerous subsequent studies with organisms of this group have shown that the importance of riboflavin in the nutrition of these organisms varies from species to species, and moreover can be deliberately altered in some cases.

On the basis of earlier work, Snell and Strong (362, 363) examined the requirement of riboflavin for the growth of eleven different species of lactic acid bacteria. The basal medium was made deficient in riboflavin by photolysis at alkaline pH. Cultures were carried through five serial transfers in any given medium to eliminate effects of carry-over from the inoculum and to show that the medium could support continued growth. The growth of *L. arabinosus*, *L. pentosus*, *B. brassicae*, *L. pentoaceticus*, *L. mannitolpoeus*, *Leuconostoc mesenteroides*, and *S. lactis* was as luxuriant in the absence of riboflavin as in its presence. Four of these species were examined and found to synthesize riboflavin. On the other hand four species, *L. delbrückii*, *L. gangori*, *B. lactis acidii*, and *L. casei* all required the addition of riboflavin and could not grow in continued sub-culture without it. Andersen and Werkman (6) found riboflavin required by an organism which produced *d*-lactic acid. Schütz and Theorell (343) found riboflavin to be present in all the species of lactic acid bacteria which they examined.

Using three species of heterofermentative lactic acid bacteria (*L. mannitolpoeus*, *L. bruckneri*, and *L. lycopersici*), Wood, Geiger, and Werkman (437) examined the interrelation of growth factor requirements. Thiamin and riboflavin stimulated the growth of *L. mannitolpoeus* and *L. lycopersici*, but neither was essential in the presence of the other. When both compounds were omitted, continued growth did not occur. In some cases when serial transfer was used to decrease the carry-over in the inoculum, the organisms adapted themselves to growth in the absence of added thiamin or riboflavin (346, 436). Wood, Geiger, and Werkman (437) emphasized that it was essential in any nutritional work to have the organisms in a proper physiological state in order to get clear-cut effects with any substance. They clearly recognized that bacteria were organisms whose properties could, within limits, change, and that the response to a given substance in the nutrient medium might therefore vary with the synthetic powers of the particular cells in the inoculum. It was not possible to generalize, in the sense of saying that a given substance was essential or non-essential for any given species. The effect of a given substance in growth showed quantitative differences and the cells of different generations, (*i.e.*, different sub-cultures) could show, within limits, nutritional differences. McIlwain (232) has also discussed the general question of the interrelation and relativeness of nutrient requirements in the case of hemolytic streptococci.

#### 4. *Propionic Acid Bacteria*

Wood, Andersen, and Werkman (436) found that riboflavin had a stimulating effect on the growth of some strains. The effect of riboflavin was relative to the other constituents of the medium, being marked when the organisms were using ammonia as a nitrogen source; apparently it was not required in amino acid media. Other cultures were trained to dispense with the need for added riboflavin when growing on the ammonia medium. Whether these organisms then synthesized riboflavin was not examined, but it seems probable that they did.

#### 5. *Luminescent Bacteria*

It was observed by Doudoroff (69) that riboflavin had an effect on the luminescence and growth of certain variants of strains of *Photobacterium phosphorescens* (13, 14) isolated from sand-dabs. When kept on yeast autolyzate agar containing 1% glycerol and 3% NaCl many of the strains dissociated producing 'dark' and 'dull' colonies. When riboflavin was added, cultures from the 'dark' or 'dull' colonies luminesced more brightly. Using a Witte peptone liquid medium, an effect of riboflavin on growth was seen. The 'dark' or 'dull' variants which responded to riboflavin by becoming luminous could not grow immediately on the Witte peptone medium unless riboflavin (at about  $5 \times 10^{-8} M$ ) was added. The 'dull' variants were stimulated in growth by the addition of riboflavin, becoming comparable with the original bright cultures, and the intensity of their luminescence increased in proportion to the amount of riboflavin added. It was concluded that the 'dull' variants had lost the ability to produce sufficient riboflavin for their needs, whereas the original bright cultures could do so. Riboflavin evidently played a part both in the growth of the organism and in its light-producing reactions. The growth response was immediate with much smaller amounts of riboflavin than were needed to restore luminescence. Some 'dull' variants were not restored to luminescence by riboflavin, however, the change in physiology here involved being clearly more far-reaching. This change is interesting, since it appears to involve a loss of synthetic power. By far the most frequently observed changes of synthesizing ability under experimental conditions involve an increase in synthetic power.

#### 6. *Synthesis by Bacteria*

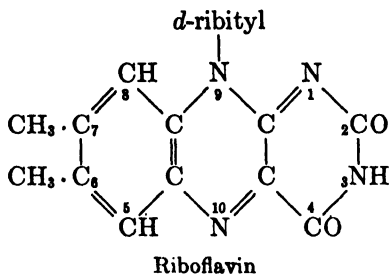
West and Wilson (409) showed that riboflavin was synthesized by *Rhizobium*, but there were indications that initiation of growth with a washed inoculum was difficult or impossible unless some riboflavin was present in the medium. This is a situation similar to the need for CO<sub>2</sub> in the initiation of growth by various organisms which produce ample CO<sub>2</sub> when once active metabolism has begun (106). West and Wilson observed that the concentration range of riboflavin and of thiamin which was effective in initiating

growth with a washed inoculum was narrow. Riboflavin was shown to be synthesized by strains of *Staph. aureus* and *Staph. flavus* (329) and by *C. diphtheriae* (83). There are other less clear observations indicating synthesis by organisms for which the substance is not a nutritional component of importance.

### 7. Specificity

The specificity of riboflavin in bacterial growth has been examined by Snell and Strong (362) using *Lactobacillus casei* and *B. lactis acidii* (both of which require riboflavin) and using a number of synthetic flavins, including:—

- I. 6,7-Dimethyl-9-(*d*-1'-ribityl)-isoalloxazine (riboflavin),
- II. 6-Ethyl-7-methyl-9-(*d*-1'-ribityl)-isoalloxazine,
- III. 6 Methyl-9-(*d*-1'-ribityl)-isoalloxazine,
- IV. 7-Methyl-9-(*d*-1'-ribityl)-isoalloxazine.



The 6-ethyl-7-methyl compound (II), in which  $\text{—C}_2\text{H}_5$  replaces  $\text{—CH}_3$  at 6 in the natural compound (I), was the only analog whose activity approached that of riboflavin itself. The 7-methyl (IV) and the 6-methyl (III) analogs showed decreased activity, in that order. Analogs having sorbityl or arabityl residues in place of the ribityl residue were completely inactive, as also were 6,7,9-trimethyl-isoalloxazine (lumiflavin) and 6,7-dimethyl-alloxazine, (in which the ribityl residue is eliminated), and riboflavin tetra-acetate. In the presence of sub-optimal amounts of riboflavin there was a slight detectable supplementary effect of certain analogs which were inactive alone. These were:

- V. 6,7-Dimethyl-9-(*d*-1'-arabityl)-isoalloxazine
- VI. 6,7-Dimethyl-9-(*l*-1'-arabityl)-isoalloxazine
- VII. 6-Ethyl-7-methyl-9-(*l*-1'-arabityl)-isoalloxazine
- VIII. 5,6-Benzo-9-(*d*-1'-ribityl)-isoalloxazine

Snell and Strong compared the activity of these riboflavin analogs with their reported activities as vitamin B<sub>2</sub> by rat assay and in Kuhn and Rudy's enzyme tests (174). The correlation was close, analogs inactive for the

bacteria being inactive for rats, with the exception of riboflavin tetra-acetate, which can be used by rats. Evidently, *L. casei* and *B. lactis acidii* are unable to hydrolyze the ester linkages whereas the rat can. There have been reports that the *l*-araboflavin (VI) was nearly as active as riboflavin in the enzyme test (174) while the *d*-araboflavin (V) was inactive. In rat growth the effects reported for these araboflavins are somewhat conflicting but never striking.

Snell and Strong (363, 364) developed the growth response of *Lactobacillus casei* to riboflavin as an assay method, and it was soon used, for example by Fraser, Topping, and Isbell (102) to assay riboflavin in the urine and tissues of normal and riboflavin-deficient dogs. The microbiological riboflavin assay has since been widely used (*e.g.*, 394).

#### IV. PYRIDOXIN

##### 1. General

Pyridoxin: 2-methyl-3-hydroxy-4,5-di-(hydroxymethyl)-pyridine, is the second pyridine derivative (the other being nicotinic acid) which has been found to have key importance in the nutrition, and hence in the metabolism, of a wide variety of organisms. It was first clearly differentiated as a member of the vitamin-B complex in the study of rat nutrition (111). Isolation, determination of structure, and synthesis quickly followed (123, 124, 146, 148, 175, 176, 177, 371). The substance has now been found to be important in the metabolism of numerous organisms, including insects, bacteria, yeasts, and molds. In Tables IX and X are collected most of the organisms for which pyridoxin has been shown to play a part in growth. As always in this field, a table tends to give too rigid and formal a picture. Some amplification and qualification is given in the succeeding sections.

##### 2. Bacteria

Pyridoxin was first implicated in bacterial nutrition by Möller (249) who showed that it was required as an essential nutrient by certain lactic acid bacteria. Möller used specimens of the substance isolated from natural sources, the findings being confirmed later with synthetic material (250, 251). Effects of pyridoxin in bacterial nutrition have since been extended to other species. There are now several bacterial species or strains known whose need for pyridoxin is very definite. How stable these nutrient requirements are is not yet determined; it has not yet been shown whether a strain which cannot normally synthesize pyridoxin can be trained to do so. However, this seems possible, since there have been found, among naturally-occurring strains, different degrees of dispensability of pyridoxin. Among the lactic acid bacteria some naturally-occurring strains have been observed which synthesize pyridoxin sufficiently slowly for a stimulation of growth to be



TABLE IX

*Organisms for Which Pyridoxin Has an Effect on Growth*

Higher animals	Pyridoxin effect	References
<b>Rat</b>	Cures an acrodynia-like dermatitis	24, 110, 113, 203
<b>Puppies</b>	Cures a microcytic hypochromic anaemia	96
<b>Insects</b>		
Mosquito larvae ( <i>Aedes aegypti</i> )	Essential nutrient	377
<i>Tribolium confusum</i>	? Essential; growth stimulation	98
<i>Ptinus tectus</i>	? Essential; growth stimulation	
<i>Sitodrepa panicea</i>	? Essential; growth stimulation	
<b>Bacteria</b>		
<b>Lactic acid bacteria:</b>		
<i>Bact. acetylcholini</i> (Keil) Si <sub>1</sub> , Si <sub>2</sub> , etc. — 8 strains	Essential nutrient	249, 250, 251
<i>Streptobact. plantarum</i> P. 24:		
<i>Bact. cucumeris fermentati</i> B <sub>1</sub> and others of Henneberg's original strains		
<i>Lactobacillus casei</i> (A.T.C.C. No. 7469)		
<i>Lactobacillus delbrückii</i> 3	" "	359
<i>Lactobacillus lactis</i> B1-1	" "	
<i>Leuconostoc mesenteroides</i>	Growth stimulant; pyridoxin slowly synthesized	
<i>Lactobacillus arabinosus</i> 17-5	Pyridoxin synthesized	
<i>Lactobacillus pentosus</i> 124-2	Pyridoxin synthesized	
<b>Streptococci:</b>		
<i>Strept. hemolyticus</i> . "Richards" (Lancefield type A)	Essential nutrient	232
<i>Strept. hemolyticus</i> . C. 203 S (Lancefield type A)	" "	277
<i>Strept. zymogenes</i> H-6095 (Lancefield type D)	" "	135, 449
<i>Strept. mastitidis</i> (Lancefield type B)	" "	449
<i>Strept. fecalis</i>	" "	342
<i>Strept. lactis</i> R. (A.T.C.C. 8043)	Prefers a more active metabolite derived from pyridoxin	353, 355, 353a
<i>Staphylococcus albus</i>	Growth stimulant	402

demonstrable, while others synthesize pyridoxin so rapidly that no further stimulus is seen on adding it to the medium. Thus Bohonos, Hutchings, and

Peterson (28) examined a number of species of lactic acid bacteria and observed varying responses to pyridoxin. For *Lactobacillus casei* (American Type Culture Collection No. 7469; *L. casei*  $\epsilon$ ; *L. helveticus*), the response varied with the age of the culture used to supply the cells for the inoculum, with the number of serial sub-cultures from the stock culture, and with the pyridoxin concentration. Part of the variation in response was due to differences in the amount of pyridoxin actually stored in the cells of the inoculum. This is an important variable which has not always been carefully controlled in growth experiments with bacteria. It is clear that this effect is different from that due to mechanical carry-over of growth factors in the inoculum, which has sometimes been a source of error. Washing of cells can mitigate this latter effect, but not that due to stored growth factors. *L. casei* is known also to store biotin, but storage of riboflavin and pantothenic acid has not been observed. The storage effect can be mitigated by using starved organisms, obtained by growing cells in a partially deficient medium, as was done for thiamin-deficient cells of *Staph. aureus* (127).

With allowance for the storage effect Bohonos, Hutchings, and Peterson showed that *L. casei*, *L. delbrückii*, and *L. lactis* required pyridoxin in the media, while *L. arabinosus* and *L. pentosus* grew just as well without added pyridoxin as in its presence. The latter organisms were shown to synthesize pyridoxin. With *Leuconostoc mesenteroides* a stimulation of growth by pyridoxin was observed, although continued sub-culture could take place on a pyridoxin-free medium, *i.e.*, synthesis took place, but not at a rate sufficient for maximum proliferation. It was shown directly that *Leuconostoc mesenteroides* only synthesized about  $\frac{1}{4}$  the amount of pyridoxin synthesized by *L. arabinosus* and *L. lactis*. Together these results provide a beautiful example of how the rate of synthesis of an essential metabolite will determine whether this substance will appear as an essential nutrient (when the rate is very slow), as a growth stimulant (when the rate is greater but still sub-optimal), or as a factor not required at all in the medium (when the rate of synthesis is so great that it is no longer a limiting condition). For organisms which require pyridoxin as an essential for growth, the rate of synthesis is effectively zero.

Another case where slow synthesis of pyridoxin appears to take place was recorded by Vilter and Spies (402) for a strain of *Staphylococcus albus*. This organism showed a stimulation of growth and acid production when pyridoxin (0.3 to 1.2  $\mu\text{g./ml.}$  medium) was added to a chemically-defined medium which already contained the two "essential" growth-factors thiamin and nicotinic acid in concentrations allowing moderate growth.

Snell, Guirard, and Williams (355) found evidence for a physiologically active substance derived from pyridoxin which was much more active for *Streptococcus lactis* R than pyridoxin itself, and Snell (353) showed that

under the conditions of heat sterilization of media containing pyridoxin, a more active compound was formed. Autoclaving pyridoxin with cystine and glycine was effective in forming a more active derivative. *Streptococcus lactis* R appears to be somewhat more fastidious in its growth requirements than some other species which require pyridoxin, and it suggests that *Streptococcus lactis* R is less able than these to synthesize a more complex substance which is the real metabolite used in some essential reaction.\*

Various species and strains of *Streptococcus* (see Table IX) require pyridoxin as an essential nutrient. The nutrient requirements of these organisms are complex, and other essential metabolites besides pyridoxin must be given them. Here it is worth stressing that, just as the requirement for a given essential metabolite as a nutrient is conditional on the rate of synthesis of this substance by the cells, so too there may be considerable interdependence in requirements for different essential metabolites, because of the general interlinking of metabolic processes in the cells. Thus a nutritional requirement for a given essential metabolite may be conditional not only on its rate of synthesis but also on the rate of synthesis of another essential metabolite.

### 3. Inter-relations of Cultural Requirements of Bacteria

Illustrative of the way in which cultural conditions may influence nutritional requirements are the results mentioned above for lactic acid bacteria (28). For these organisms, the pyridoxin concentration required varied with the oxygen tension of the gas-phase in which the organisms were grown; the lower the partial pressure of oxygen, the more pyridoxin was required. Thus again, a nutrient requirement was not an absolute one but was relative to the functioning of other metabolic processes. The results may indicate either (1) that the cells under lowered oxygen tensions were carrying out certain reactions involving pyridoxin on a greater scale than occurs when oxygen is freely available, and thus required more pyridoxin for a given amount of cell proliferation; or, (2) that a certain rate of pyridoxin synthesis was possible under high oxygen tensions which was not maintained under the lower oxygen tensions, the rate then becoming insufficient to supply enough pyridoxin for growth requirements.

An example of the latter possibility was found by Richardson (293) with *Staphylococcus aureus*. It was found (see section X) that uracil was an essential nutrient for this organism only under anaerobic conditions. When growing aerobically the organism was able to synthesize adequate amounts of uracil.

Pappenheimer, Jr., and Hottle (277) observed another relation between pyridoxin requirements and cultural conditions with *Streptococcus hemo-*

*Note added in proof.*

\*Recent work indicates that pyridoxal and pyridoxamine are the more active higher-stage metabolites derived from pyridoxin (353a, 124a). One function of pyridoxal (phosphorylated) is as the co-enzyme of tyrosine decarboxylase (109a).

*lyticus* (Group A) C 2035, the pyridoxin required varying with the CO<sub>2</sub> tension of the gas-phase. In absence of pyridoxin, no significant growth occurred at a CO<sub>2</sub> tension of 0.4 mm. Hg, but at 8 mm. tension of CO<sub>2</sub> the growth was about half that of the maximal growth which occurred when pyridoxin was present in optimal amount (about 2.0 µg./ml. medium).

A close connexion between the requirements of the organism for adenylic acid and the CO<sub>2</sub> tension was also found (see Section X, 4 and Table XXV). In general, suitable pressures of CO<sub>2</sub> could off-set lack of adenylic acid and to some extent lack of pyridoxin with *Streptococcus hemolyticus* C 2035, and decreased oxygen pressure (anaerobiosis) also decreased the adenylic acid effect. Further investigation of these extremely interesting relations between pyridoxin, adenylic acid, oxygen, and carbon dioxide may provide clues to the metabolic roles of both pyridoxin and carbon dioxide in bacterial growth, both of which are at present obscure.

#### 4. Yeasts

Pyridoxin plays a part in the growth requirements of certain yeasts (74, 340, 354, 425) and it appears probable that for this type of micro-organism it is an essential metabolite which is fairly easily synthesized, as effects with pyridoxin are confined to growth stimulation. Thus Williams, Eakin, and Snell (425) found that continued growth of certain yeasts could take place in a medium containing no pyridoxin, although marked stimulation was seen when pyridoxin was added. Under the conditions of test the presence of pyridoxin (+ β-alanine) rendered the growth response in a biotin titration much more sensitive. Williams (423) considers it probable that pyridoxin is synthesized by those yeasts which give continued growth in a medium lacking pyridoxin and which show stimulatory effects, and increased sensitivity to other nutrilites, on its addition. When pyridoxin evokes an increased rate of growth, it is clear that the rate of its synthesis must be a limiting factor in the growth processes, and it is understandable why the addition of preformed pyridoxin, by removing this limiting condition, can cause the response to other nutrilites to be more sensitive, as in the biotin response noted by Snell, Eakin, and Williams (354).

Leonian and Lilly (201) examined 10 strains of *Saccharomyces cerevisiae* for the effects on 72 hr. growth (25°) of omission of various vitamins from the media; none of the strains showed decreased growth on omission of pyridoxin. Marchant (245) had strains which were much more sensitive to pyridoxin, and presumably were less efficient synthesizers of it than the strains examined by Leonian and Lilly. Two yeasts, *Sacch. hanseniaspora valbyensis* and yeast 2375, showed a marked stimulation with pyridoxin which could, for these two yeasts, replace a "bios VII" solution. A strain of *Sacch. cerevisiae* has been trained to grow on a completely synthetic medium without added vitamins; the yeast then synthesized thiamin, riboflavin,

pyridoxin, nicotinic acid, biotin, pantothenic acid, and inositol (200). The dependence of growth on inter-relationships between the various essential metabolites has been studied (425), and the point may be emphasized that the amount of one essential metabolite may greatly determine the degree of dispensability of another essential metabolite.

### 5. Roots of Higher Plants

Stimulation of the growth of isolated roots of higher plants has been observed (see Table X). It would appear that pyridoxin is normally synthesized in the green leaf and transmitted to the roots (313). The specificity of pyridoxin in isolated root growth (300) is analogous to the specificity in the growth response for other types of organism (see Table XI).

### 6. Lower Fungi

Several lower fungi have already been found to require pyridoxin for growth, while in some groups related organisms have not required it as a nutrient, probably being able to synthesize it for themselves.

Robbins and Ma (316) found it a nutritional requirement of *Ceratostomella ulmi*; Fries (103) found other Ascomycetes of the genus *Ophiostoma* (*Ceratostomella*) for which it was indispensable (*O. fagi*, *O. piliferum*, *O. multiannullatum*), while for other species (*O. coerulium* and *O. quercus*) it was a growth stimulant, although not indispensable. The latter species probably synthesize pyridoxin at a sub-optimal rate. Other species showing a growth-stimulant effect with pyridoxin have been noted (318).

Very interesting observations on differences in synthetic ability among X-ray induced mutants of *Neurospora crassa* were observed by Beadle and Tatum (12). Mutants were found which grew normally on a complete (complex) medium but which gave practically no growth on a simple chemically-defined medium of mineral salts and sucrose, although this was adequate for the normal organism. Failure to grow on the chemically-defined medium was shown to be due to the need for certain growth factors. It was shown that three mutants had lost the ability to synthesize *p*-aminobenzoic acid, two to synthesize the thiazole portion of thiamin, and one to synthesize pyridoxin. The normal organism from which the mutants were derived could synthesize all three vitamins. A single gene difference apparently differentiated the mutant unable to synthesize pyridoxin from the parent organism.

A further extension of these observations has shown that it may be possible to by-pass the genetically blocked pyridoxin-synthesizing reaction in a mutant of this type by a change in the nutrient environment (374). When the pH of the medium was 6 or over, and only ammonium salts were used as source of nitrogen, the ability to synthesize pyridoxin reappeared. Nitrate, nitrite, amino acids, and amides could not be used in place of ammonia.

These conditions of pH and N source were very specific, since the new conditions only permitted pyridoxin synthesis. The mutants requiring thiamin and *p*-aminobenzoic acid were not able to synthesize these compounds

TABLE X

*Organisms for Which Pyridoxin Has an Effect on Growth*

Lower fungi	Pyridoxin effect	References
<i>Ceratostomella ulmi</i>	Essential nutrient	316
<i>Graphium ulmi</i>	" "	49
<i>Ophiostoma</i> ( <i>Ceratostomella</i> ):		
<i>O. fagi</i>	Essential nutrient	103
<i>O. piliferum</i>	" "	
<i>O. multiannulatum</i>	" "	
<i>O. coerulium</i>	Growth stimulant	
<i>O. quercus</i>	" "	
<i>Ceratostomella leptographioides</i>	" "	318
<i>Polyporus versicolor</i>	" "	
<i>Neurospora crassa</i> normal	Pyridoxin synthesized	12, 382
" " X-ray mutant;	Essential nutrient or	12, 374, 382
"pyridoxinless"	different nutrient conditions required for synthesis	
Yeasts		
<i>Saccharomyces hanseniaspora valbyensis</i>	{ Pyridoxin = Bios VII to ensure good crop }	245
Yeast 2335		
<i>Sacch. galactosus</i>	No stimulation by pyridoxin	201
<i>Sacch. cerevisiae</i> , 10 strains	No stimulation by pyridoxin	
"Trained yeast"	Synthesized pyridoxin	200
<i>Sacch. cerevisiae</i>	Growth stimulant	{ A 74, 340
" " various strains: { B	Not stimulated by pyridoxin	
" " { C	Growth stimulated by thiamin and pyridoxin	
Higher plants, isolated roots		
Tomato	Probably essential nutrient	32, 60, 313
" (inbred)	Pyridoxin less necessary ? synthesized	
Carrot, <i>Datura</i> , sunflower etc.	Probably essential nutrient	

under the conditions which allowed the "pyridoxinless" mutant to synthesize pyridoxin. It was suggested that the new nutrient requirements of the mutant, in order for it to synthesize pyridoxin, reflected the biochemical

changes produced by the X-irradiation, *i.e.*, an alteration in the nitrogen metabolism involved in pyridoxin synthesis, this being a function of pH. The interplay of genetic control of metabolic reactions and nutritional conditions is very beautifully illustrated by these mutants. The example subsumes a number of the themes of this article. Here it is clearly seen that the nature of the nutritional requirement of a given organism is complementary to the synthetic abilities of that organism; certain metabolic reactions are general and fundamental, and the means of carrying them out are supplied

TABLE XI  
*Biological Specificity of Pyridoxin*

Compound:	Rat (393)	Lactic acid bacteria (28, 251)	<i>Cerato- stomella ulmi</i> (316)	Tomato root (300)
	RELATIVE ACTIVITIES:			
Pyridoxin:				
2-Methyl-3-hydroxy-4,5-bis-(hydroxy methyl)-pyridine	1	1	1	1
2-Ethyl-3-hydroxy-4,5-bis-(hydroxy- ethyl)-pyridine	0			1
Derivatives of 2-methyl-pyridine:				
3-Hydroxy-4,5-bis-(acetoxymethyl)-	1	0.8-1.0	1	1
3-Acetoxy-4,5-bis-(acetoxymethyl)-	1	0	1	1
3-Hydroxy-4-methoxymethyl-5-hy- droxymethyl-	0.2	0.3-0.4		
3-Hydroxy-4-ethoxymethyl-5-hy- droxymethyl-	0.2	0.3		
3-Methoxy-4,5-bis-(hydroxymethyl)-	0.02			
3-Hydroxy-4,5-epoxydimethyl-	0.02	0.2-0.3		
3-Hydroxy-4,5-bis-(bromo-methyl)-	—	0.6-0.8		
3-Hydroxy-4-methyl-5-hydroxymethyl-	0	{ 0.03 (?) <0.02		

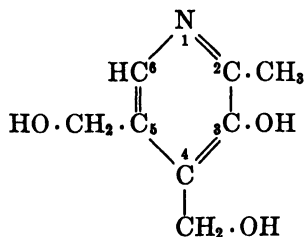
Inactive for rat and *Lactobacillus casei*: 3-Amino-4-ethoxymethyl-5-aminomethyl-2-methyl-pyridine; 3-Amino-4-hydroxymethyl-5-aminomethyl-2-methyl-pyridine; 3-Amino-4-bromomethyl-5-aminomethyl-2-methyl-pyridine; 3-Hydroxy-4,5-dimethyl-5-aminomethyl-2-methyl-pyridine; Lactone of 3-hydroxy-4-hydroxymethyl-5-carboxy-2-methyl-pyridine; Lactone of 3-amino-4-hydroxymethyl-5-carboxy-2-methyl-pyridine.

either by the environment (nutrients) or by the organism. Furthermore, the possibilities of synthesis themselves are conditioned by the environment, as shown with *Neurospora* "pyridoxinless" mutants. With bacteria, the biological means whereby daughter cells possess the main characters of the parent cells is much less highly differentiated and less sharply defined than in higher types of organism. Possibly for this reason, in bacteria the chemical composition of the environment is able to affect more directly the functioning of the cells, resulting in the phenomena of adaptive enzyme

formation and easily alterable processes of synthesis. In higher types of organism, where an apparatus of control by genes exists, the controlling parts of the organisms are already becoming insulated from simple and direct chemical effects of the environment. With the group of X-ray induced mutants of *Neurospora* one gene appears to control pyridoxin synthesis. Such mutations when occurring naturally would tend to lead to a more parasitic habit for those mutants with more restricted synthetic powers. They could only survive in an environment where, for example, pyridoxin was already available, or where conditions were suitable for its synthesis,—conditions more specific than those required by the normal forms. This restriction would then condition the selection of other mutants which might arise in the new environment.

### 7. Specificity

Observations on the activity of pyridoxin and closely related compounds with a variety of different types of organism are collected in Table XI. The results show the high biological specificity of the pyridoxin structure



Pyridoxin

and are indirect evidence for a common metabolic reaction involving pyridoxin. The slight differences in availability, such as the inactivity of the triacetate for *L. casei* compared with its ready availability for the rat, *Cerastomella ulmi*, and tomato root, probably reflect the ability of the latter organisms to derive the master compound from the related one.

### 8. Metabolism

From the wide range of organisms of very different types, including higher animals and plants, yeasts, bacteria, and some fungi, into whose metabolism pyridoxin enters, in ways at present unknown, it is clear that the metabolic reactions involved must be fundamental to living matter. The existence of organisms which require pyridoxin as nutrient, because they cannot synthesize it, presents an opportunity to study this key role through examination of the effects of pyridoxin deficiency.



## V. GLUTAMINE

1. *Glutamic Acid and Amino Acid Requirements of Bacteria*

Some species of bacteria can utilize ammonia as sole nitrogen source for the synthesis of all their nitrogen-containing substance, including protein, while other species show lesser synthetic ability and require one or more amino acids, which are used in part as structural units for protein synthesis. Many species and strains of bacteria show considerable adaptability in their powers of amino acid synthesis, and it has been found relatively easy to train organisms to dispense with the need for certain amino acids by becoming able to synthesize them. Glutamic acid has been included in amino acid mixtures used in compounding media of known composition for cultivating numerous species of bacteria. With the above qualifications as to adaptability in synthesis, glutamic acid has in some cases been found indispensable; *e.g.*, Woolley and Hutchings (449) found it indispensable for strain H-6905 of *Streptococcus zymogenes*. From numerous similar cases it has been concluded that the glutamic acid, with other amino acids required by the given organisms, has been used at least in part as a structural unit in protein synthesis. These introductory remarks are needed in order to set in perspective the findings with glutamine and glutamic acid which are to be discussed.

2. *Glutamine in Bacterial Nutrition*

Glutamine was first implicated in the growth requirements of micro-organisms when it was identified as the active labile substance present in a variety of sources, which was required for growth of certain strains of hemolytic streptococci on a deficient medium (242).

Glutamine has now been shown to have similar growth-promoting effects with a number of other bacterial species: *Streptococcus hemolyticus*, majority of Group A strains (92); *Strept. hemolyticus*, C 2035 (277); *Streptococcus viridans* (92); some strains of *Lactobacillus casei* (85, 286) and other lactic acid bacteria (286); a strain of *Diplococcus pneumoniae* Type I (92); a strain of *B. anthracis* (92). In order to appreciate the somewhat special role of glutamine as a growth factor, some detailed discussion of the experimental conditions of testing is needed. This discussion illustrates once again the need to study the conditions under which multiplication is initiated and continued in relation to the synthetic powers, metabolic and nutritional requirements of the bacteria.

The test organism used by McIlwain, Fildes, Gladstone, and Knight (242) was the well-known "Richards" strain of *Strept. hemolyticus* maintained at high virulence by repeated mouse-passage and kept in the intervals in rabbit blood. These were conditions calculated to preserve the character of the

test organism both as regards nutritional requirements and pathogenic properties. Cultures were transferred to nutrient agar as required. After a variable number of sub-cultures on agar the coccus was of no use as test-object since it became able to grow on the basal medium alone, without the active tissue-extract it required when fresh from animal passage. The alteration in nutrient requirements was ascribed to the development of the power to synthesize the tissue-extract factor, which power the organism lacked when maintained in blood. Because of this power to change its synthetic ability, short-period growth tests were used and the test-organisms were recovered from blood at short intervals. Using organisms thus maintained in a highly fastidious state as regards nutritional requirements, it was eventually found that the substance present in tissue extracts which was required by these organisms in order to proliferate was glutamine.

Glutamic acid in much higher concentration ( $M/50$ ) produced about the same effect as  $M/5000$  ( $30\text{ }\mu\text{g./ml.}$ ) glutamine; aspartic acid was ineffective. Further analysis of the effects of glutamine and glutamate were made (92) by varying the size of the inoculum, whereby differences in utilizability were emphasized. The training of the organisms to dispense with the need for glutamine was also studied.

The general conclusion drawn was that those cultures which required glutamine did so because they were unable to synthesize it sufficiently rapidly to satisfy essential metabolic reactions in which glutamine played a part. The pathogenic "Richards" strain maintained by animal passage could be looked upon as one which had, during its evolution, become modified by loss of certain functioning enzymes concerned with glutamine synthesis. The loss was not absolute, since when the inoculum was large enough glutamine appeared to be synthesized in sufficient amount, especially when glutamate and iron were present in adequate concentration. The training to dispense with added glutamine was interpreted as due to an increased concentration per cell of the enzymes involved in glutamine synthesis from glutamate.

### 3. Initiation of Bacterial Proliferation

An effect of glutamine was observed which was different from that observed with many other growth factors. For example, with *Proteus vulgaris* and a suitable deficient medium, the mass of growth is proportional to the amount of nicotinic acid present (88); a similar relation is found with other essential nutrients and forms the basis of many microbiological assay methods. But with glutamine this type of proportionality was not observed; when sufficient glutamine was present to initiate growth, the mass of growth became, with time, not limited by the initial glutamine concentration. It appeared that when growth was started with a minimal amount of glutamine, more glutamine was synthesized from a constituent of the medium.

This synthesis, however, did not depend upon an alteration in the powers of the cocci in the medium; it appeared that the normal untrained (*i.e.*, to synthesize glutamine) cocci could only start to multiply when in the presence of sufficient glutamine, and that when proliferation had begun it proceeded just as in the presence of excess glutamine. Preformed glutamine was thus essential for the initiation of multiplication. This need for a special substance for the *initiation* of growth, which the organisms, when once multiplying, can more or less efficiently make for themselves, is an interesting special case of which only a few examples have been studied. The need for CO<sub>2</sub> in the initiation of growth of many heterotrophic bacteria which when actively metabolizing produce large quantities of CO<sub>2</sub> appears to be similar (106). Both cases emphasize the need to consider microbial proliferation from the point of view of rates of synthesis of essential metabolites; to focus attention on nutritional needs alone hampers biological interpretation.

The glutamine effect has been demonstrated with numerous strains of streptococcus (92). The need for glutamine was relatively most marked with the Group A strains, and least in Group B, while of other groups some were sensitive to glutamine and others not. Four strains of *Streptococcus viridans* required glutamine while 3 strains of *Strept. fecalis* were unaffected by it. The strain of Group A *Strept. hemolyticus* studied by Pappenheimer and Hottle (277) required glutamine, and later the glutamine requirements for massive growth were studied quantitatively (21, 22).

#### 4. Glutamine in the Growth of Lactic Acid Bacteria

Feeney and Strong (85) investigated the growth response of *Lactobacillus casei* on a basal medium deficient in pantothenic acid which had been designed for pantothenic acid assay. It was found that the basal medium was inadequate to reveal the maximum effect of additions of pantothenic acid, growth being limited by deficiency of other factors, and a number of substances were found which markedly influenced the growth of the test organism under these conditions. These included glutamine, glutamic acid, and asparagine. Glutamine was stimulatory at very low concentrations; 0.1 µg./ml. medium showed a detectable effect, with progressively greater stimulation with increasing concentration. The effect of glutamic acid was observable at 100 µg./ml. medium, and of asparagine at 10 µg./ml. medium.

A comparative study of these substances and of their effects on one another showed that the relative effects varied at different stages of growth, that of glutamine being more marked early in the growth period (*cf.* 92). The heaviest growth at 24 hours was produced by asparagine and glutamic acid together. In contrast to the stimulatory effects of these three structurally related substances, aspartic acid was strongly inhibitory; it nullified the stimulatory effect of asparagine, but not of glutamine or glu-

tamic acid. As little as 0.1  $\mu$ g. glutamine per ml. counteracted the aspartic acid inhibition. The following conclusions were drawn: glutamine is to be looked upon as the key substance (essential metabolite) and can be synthesized by the organism, large amounts of glutamic acid aiding the synthesis. This supports the conception of Fildes and Gladstone (92). Asparagine can in some measure replace glutamine, but not so efficiently, possibly by providing easily available amide groups for the formation of glutamine from glutamic acid. The aspartic acid inhibition might be due to competitive inhibition of an enzyme concerned in the transfer of the asparagine amide group to glutamic acid; cf. Gladstone's antagonistic amino acid pairs (105). Asparagine might have other functions besides assisting in glutamine formation.

Pollack and Lindner (286) also observed stimulatory effects of glutamine and glutamic acid on a number of strains of lactic acid bacteria. The effects were recorded in terms of the mass of cells formed after 13 hours' incubation, on a basal medium of known composition, including aspartic acid and asparagine but not glutamic acid. With all 9 organisms there was practically no growth without glutamine and a definite response to 0.2 to 0.6  $\mu$ g. glutamine/ml. medium in the 13 hours' test. *l* (+)-Glutamic acid also had a strong growth-promoting effect but the amounts required varied for different organisms. For 5 of the 9 organisms glutamic acid was as effective as glutamine, while the remaining 4 organisms required at least 11 times more glutamic acid than glutamine for the production of equivalent growth. Pollack and Lindner included a strain of *Lactobacillus casei* in their series, and under the conditions of test glutamine and glutamic acid were equally effective. It is probable that cells used for inocula from different cultures will vary in their abilities to use glutamic acid or glutamine, in the same way as already mentioned for *Strept. hemolyticus* (92, 242). Variations in the ability of different lots of cells to make glutamine from glutamic acid would account for the different behavior of different cultures of the same strain of organism.

Pollack and Lindner suggested that glutamine and glutamic acid were both used for a similar purpose but that different strains differed in their abilities to use the two substances. This is in conformity with the views already mentioned (92, 85). They also observed that addition of  $\text{NH}_3$  to the medium did not increase the effectiveness of glutamic acid with the 4 strains for which glutamine was most potent. From this, and because "the basal medium contained no source of ammonia other than amino acids and other organic compounds" [including asparagine-K], Pollack and Lindner concluded that it was "unlikely that glutamic acid was being converted to glutamine." But this does not follow; there is no evidence that the organisms could not de-aminatate an amino acid or use the amide group of asparagine,

as Feeney and Strong suggested. And the failure of (ammonia + glutamic acid) to be as effective as glutamine with certain strains is entirely consistent with the view already noted, that such strains as required glutamine lacked the ability to convert glutamic acid to glutamine because of a deficiency of the required synthesizing enzyme, not because of lack of ammonia. The observations of Pollack and Lindner are consistent with the interpretation above, namely that glutamine is the required essential metabolite, and that the use of glutamic acid depends on the ability of any given cells to make glutamine from it. The enzymes required for this synthesis appear to be very easily acquired.

### 5. Specificity

McIlwain (231) examined the structural specificity of the glutamine effect with *Streptococcus hemolyticus*, using the same strain and conditions for maintaining maximum sensitivity to glutamine as previously described (92, 242). An inoculum of only 1000 cocci was used. A wide variety of glutamine analogues and derivatives was found incapable of replacing glutamine, whose function was clearly very specific. Furthermore the test organism was presumably incapable of converting any of the compounds tested into glutamine, including such relatively simple operations as forming the  $\gamma$ -amide of glutamic acid (glutamine), hydrolyzing an *N*-acetyl, *N*-leucyl or *N*-cysteyl grouping, or hydrolyzing the peptide link between the —COOH group of glutamine and the amino group of glycine, glutamic acid, or cysteine, since it could not utilize the corresponding peptides, nor could the organism open the rings of pyrrolidone- $\alpha$ -carboxylic acid or glutamic acid imide, by addition of  $\text{NH}_3$  or  $\text{H}_2\text{O}$  in the appropriate positions.

From the inability of *Strept. hemolyticus* to utilize glutamine peptides (e.g., *l*-leucyl-*d*-glutamine, cysteylglutamine) McIlwain (231) suggested that the organism had no enzyme capable of freeing glutamine from these peptides, and then, from the reversibility of enzyme systems, argued that it was also incapable of building up glutamine into normal peptides, as part of the synthesis of essential protein. But this conclusion does not appear to be valid. Kurssanov (179) has discussed the problem of the ways in which a given reversible enzyme may exercise hydrolytic or condensation functions depending upon local conditions made possible by the micro-heterogeneity of the cell. And it seems quite possible, on grounds of enzyme specificity, that the cells of *Strept. hemolyticus* might lack enzymes to split the particular peptides tested by McIlwain, while possessing enzymes capable of utilizing glutamine for the synthesis of other peptides essential to the cells. McIlwain concluded that the function of glutamine might be concerned with ammonia transfer. It was shown, however, that neither ammonium pyrrolidone- $\alpha$ -carboxylate (the product of breakdown of glutamine in aqueous solution)

nor glutamic acid (the product of the action of glutaminase on glutamine) were active. But, again following Kurssanov, the simple argument from enzyme reversibility is not conclusive. The direction of action of a reversible enzyme may be conditioned by the consequences of the micro-heterogeneity of the cell or by coupled energetic reactions.

Pollack and Lindner (286) favoured the utilization of glutamine and glutamic acid for protein synthesis. They calculated from the yield of cells per unit weight of glutamine or glutamic acid used that there could be about 10–20% of glutamyl residues in the bacterial protein formed (assuming complete utilization), which is of the order found for the glutamic acid content of various proteins. The glutamic acid or glutamine requirements of the bacteria were of the order of magnitude required to give this figure.

#### *v. Conclusion*

Clearly further work is required before the function of glutamine is understood. It is clear that the nutritional need for glutamine is relatively easily overcome by the organisms examined hitherto. The general indication is that this takes place by an enzyme adaptation, leading to synthesis of glutamine by organisms which before training were unable to do this at sufficient rate.

### VI. PANTOTHENIC ACID AND $\beta$ -ALANINE

#### *1. Steps in the Recognition of Nutritional Importance*

Both pantothenic acid and  $\beta$ -alanine, which forms part of the pantothenic acid molecule, were recognized as important in the growth processes of living matter through nutritional studies with micro-organisms. Pantothenic acid itself is the first substance of general metabolic importance and of a type of structure new to biochemistry to be discovered in this way, thus reversing the order of events which had pertained until then. Methionine, which was also discovered during an investigation of bacterial nutrition (253) is excepted from the previous statement since it belonged to the already well-known class of  $\alpha$ -amino acids.  $\beta$ -Alanine was known to biochemistry as part of the peptides carnosine and anserine, but was not known to have any particular metabolic importance. As will be seen, the growth-promoting effects of  $\beta$ -alanine are probably due to its forming part of pantothenic acid.

It will be interesting, when a new history of biochemistry comes to be written, to trace the ways in which the social and economic importance of "yeasts" has stimulated, directly or indirectly, much scientific investigation which has had a major effect in developing our understanding of the fundamental biochemistry of living matter.

The history of the discovery of pantothenic acid has been well documented elsewhere (424).

The first indications of a 'single effective nutrilité' for yeast growth were observed by Williams and co-workers (426, 432) in studies of the yeast "bios" problem.  $\beta$ -Alanine was found to be a yeast nutrilité (430) before it was known to be part of the pantothenic acid molecule.

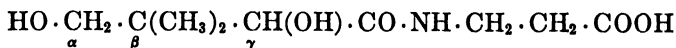
Various lines of study concerning growth factors for lactic acid bacteria, *C. diphtheriae*, hemolytic streptococci, as well as for vitamin B group deficiencies in chicks (anti-dermatitis factor) and rats (liver filtrate factor) came together when concentrated preparations of pantothenic acid became available for testing. It was soon shown that the active substance of the pantothenate concentrates appeared to be the common active factor required by all organisms. Snell, Strong, and Peterson (360, 361) showed that pantothenate concentrates of Williams replaced the growth factor preparations from liver which they had found essential for the growth of certain lactic acid bacteria and that the properties of the two active principles were the same, leaving no doubt that the active substance in the liver preparation was pantothenic acid.

Mueller (257, 265) found that  $\beta$ -alanine was a growth factor required by certain strains of *C. diphtheriae*, and later Mueller and Klotz (266) found that the "Allen" strain of *C. diphtheriae* responded more readily to pantothenic acid concentrates than it did to the hydrolytic products of the latter, or to an approximately equivalent quantity of  $\beta$ -alanine, R. J. Williams having suggested that pantothenic acid was a compound of  $\beta$ -alanine and a hydroxy acid in amide linkage. This was then demonstrated directly (404).

Woolley and Hutchings (448) found that an alkali-labile factor in liver required by certain hemolytic streptococci was replaceable by pantothenic acid concentrates, and that the two portions of the alkali-split molecule (inactive) could be recombined to give the active compound by methods which assumed that Williams' suggestion of an amide linkage between  $\beta$ -alanine and an hydroxy acid was correct, thus giving strong evidence in support of this hypothesis. Before the actual structure of the hydroxy acid portion was established, synthesis of pantothenic acid was attempted (376, 448) by coupling  $\beta$ -alanine in amide linkage with certain hydroxy acids which it was thought might be the hydroxy acid portion of pantothenic acid. These substances had some, but only very low, activity, showing that the correct hydroxy acids had not been chosen, although the slight activity observed supported the main conception.

The structure of the non-nitrogenous part of pantothenic acid was reported as an  $\alpha$ -hydroxy- $\gamma$ -lactone (247) and finally found to be  $\alpha$ -hydroxy- $\beta$ , $\beta$ -dimethyl- $\gamma$ -butyrolactone (pantoic lactone) (372, 427). Synthe-

sis and the condensation of pantoic lactone with  $\beta$ -alanine to give pantothenic acid itself, with resolution to give the naturally occurring optical isomer followed (373, 428). Throughout the work which culminated in the determination of the structure of pantothenic acid and its synthesis, the determination of biological activity by microbiological assay methods played an important part (441). Pantothenic acid has the structure:



$\alpha, \gamma$ -dihydroxy- $\beta, \beta$ -dimethyl-buteryl residue  
(Pantoic acid)

$\beta$ -alanine residue

The role that pantothenic acid plays in the nutrition of higher animals will not be discussed here; for this the review by Williams (424) should be consulted. Attention here will be concentrated on its role in the growth of micro-organisms.

## 2. Pantothenic Acid in the Growth of Bacteria

a) *General.* For some bacteria, pantothenic acid is an essential nutrient, for others only a part of the molecule ( $\beta$ -alanine or pantoic lactone; the free pantoic acid lactonizes easily and usually this part of the molecule is used in growth tests as the lactone) is essential as a nutrient. Still other bacteria have been found to synthesize it readily (386). The whole picture of pantothenic acid and its part in bacterial growth is most readily interpreted as that of an essential metabolite required in some fundamental processes common to all these organisms, which among themselves show differing powers of synthesis of this essential substance. This is reflected in their nutritional requirements.

Table XII records a group of bacteria for which pantothenic acid itself has been found to be an essential nutrient. In reading Table XII, it must always be borne in mind that the findings refer only to the actual cultures examined. It is not permissible to generalize and to conclude that all strains under a given specific name will have the same nutritional requirements. The nutritional requirements are always relative to the synthesizing ability of the cells of the inoculum, and this may differ from strain to strain within a species, or even from culture to culture of the 'same' strain, depending on the cultural conditions, including the presence of other growth factors.

b) *C. diphtheriae.* Within the species *C. diphtheriae*, different strains show differing requirements for pantothenic acid, as shown in Table XIII. There seems little doubt that within the *C. diphtheriae* species it will be found that all the organisms require pantothenic acid as an essential metabolite. In the cases deliberately examined which could utilize  $\beta$ -alanine it was found



TABLE XII

*Bacteria for Which Pantothenic Acid is Required as Nutrient*

	References
Lactic acid bacteria:	
<i>Lactobacillus casei</i>	173, 178, 190, 247, 283, 361, 362a
<i>Bacillus lactic acidii</i>	
<i>Lactobacillus arabinosus</i>	
“ <i>pentosus</i>	
“ <i>delbrückii</i>	
<i>Bacillus brassicae</i>	
<i>Streptococcus lactis</i>	
<i>Leuconostoc mesenteroides</i>	
<i>Streptobacterium plantarum</i>	
Propionic acid bacteria:	
<i>Propionibacterium pentosaceum</i>	173, 387
“ <i>jensenii</i> —1	
“ <i>jensenii</i> —29	
“ <i>thonii</i> —15	
“ <i>petersonii</i> —20	
“ <i>technicum</i> —22	
Streptococci:	
<i>Streptococcus hemolyticus</i> “Richards” (Lancefield A)	230
“ “ Dochez NY5	376
“ <i>epidemicus</i> , strains X 40, X 32, C 108, W 116-7	448
<i>Streptococcus pyogenes</i>	
“ <i>zymogenes</i>	
“ <i>fecalis</i>	
“ <i>salivarius</i>	
<i>Pasteurella</i> ; hemorrhagic septicemia, 13 strains	17, 18, 20
<i>Proteus morganii</i>	279, 281
<i>Pneumococcus</i> ; strains of Types I, II, V, VIII	291, 292
<i>Clostridium tetani</i>	267, 268
<i>Clostridium welchii</i> ( <i>perfringens</i> ) SR 12	380
<i>C. diphtheriae</i> ; certain <i>gravis</i> strains	83
<i>Shigella paradysenteriae</i> (Flexner), certain strains	403

TABLE XIII

*Pantothenic Acid in the Nutrition of C. diphtheriae*

Strain	Nutrient required		References
6 Exacting <i>gravis</i> strains	Pantothenic acid	Pantothenic acid not synthesized	83
Certain <i>intermedius</i> strains	Pantothenic acid	Pantothenic acid not synthesized	119
Non-exacting <i>gravis</i> strains	$\beta$ -Alanine	Pantothenic acid synthesized	83, 84
<i>Mitis</i> strains	$\beta$ -Alanine	Pantothenic acid synthesized	83, 84
Allen strain ( <i>mitis</i> )	$\beta$ -Alanine	Pantothenic acid probably synthesized; not yet shown in all cases.	257, 265
<i>Intermedius</i> strains	$\beta$ -Alanine		
Certain “non-exacting” strains	Amino-acid-mineral medium		

that pantothenic acid was in fact synthesized (83). Those exacting *gravis* strains which required the intact pantothenic acid molecule were unable to unite the two portions of the substance when given them together, since the hydrolysis products of pantothenic acid were inactive. A wide degree of difference in synthetic ability among strains of *C. diphtheriae* appears common, since different nutritional requirements among a group of strains with respect to other substances (*e. g.*, pimelic acid) have also been observed. Mueller (263) found that only the naturally-occurring *l*-carnosine (*N*- $\beta$ -alanyl-histidine) and not the *d* form could be used by *C. diphtheriae* as a source of  $\beta$ -alanine. The organism apparently had enzymes capable of splitting the one form but not the other.

*c) Pasteurella.* Berkman (17) found *Pasteurella* strains which required pantothenic acid and needed the intact molecule; the two components together could not be used.

### 3. Bacteria Using Portions of the Pantothenic Acid Molecule

There is a group of bacteria for which the need for pantothenic acid is not so imperative as for those listed in Table XII. In some cases a stimulation of growth by pantothenic acid has been observed; presumably these organisms under the conditions of test were able to synthesize pantothenic acid only at a sub-optimal rate. In other cases one or other portion of the pantothenic acid molecule has been found to be a growth factor, the presumption being that, given the one portion, the organism can complete the synthesis of the whole molecule (see Table XIII). With some strains of *Brucella*, Koser, Breslove, and Dorfman (166, 167) observed a growth-promoting effect with pantothenic acid, although with several of the strains growth took place without added pantothenic acid. With one strain, *Brucella suis* 1662, the following effect was observed:

- (a)  $\beta$ -Alanine or pantoic lactone separately accelerated growth.
- (b)  $\beta$ -Alanine + pantoic lactone together, greater acceleration than (a).
- (c) Intact pantothenic acid, greater acceleration than (b).

Other strains showed a similar but less marked effect.

McCullough and Dick (227) have reported similar findings with *Brucella suis*, and later (228) observed acceleration of growth of certain *Br. abortus* strains.

Numerous strains of *C. diphtheriae* of *intermedius* and *mitis* and some *gravis* types need  $\beta$ -alanine (83, 84, 257, 265) and it has been shown (83) in some cases that these strains do synthesize pantothenic acid. They are therefore able to synthesize pantoic acid and join it to the  $\beta$ -alanine.

Two cases have been recorded where an organism requires as nutrient only the pantoic acid (as lactone) portion of pantothenic acid, presumably being able to synthesize  $\beta$ -alanine effectively. These organisms are: *Acetobacter suboxydans* (A.T.C.C. No. 621) (392) and a strain of *Strept. hemolyt-*

*icus*, H 69 D (439). In the latter case pantothenic acid was more effective than the lactone, again supporting the idea that pantothenic acid was the ultimate metabolite required by the organism. No cases have been recorded of *Streptococci* which can utilize  $\beta$ -alanine only. All these observations are paralleled by the utilization of the two portions of thiamin, discussed in Section II, 5.

#### 4. Specificity

Although pantothenic acid appears to be relatively highly specific, some cases of a much lower activity by certain analogs have been observed. The specificity of the  $\beta$ -alanine portion appears to be greater than that of the pantoic acid portion. Condensation of  $\alpha$ -alanine,  $\beta$ -aminobutyric acid, aspartic acid and lysine, instead of  $\beta$ -alanine, with pantoic lactone, gave biologically inactive products (405); and Kuhn and Wieland (178), using *Streptobacterium plantarum*, found the leucine-pantoic lactone condensation product to be inactive. The difference in availability of  $\beta$ -alanine from *l*-carnosine (available) and *d*-carnosine (not available) observed by Mueller (263) depended on the ability of the *C. diphtheriae* strain "Allen" to split only the *l*-form.

Table XIV records the effects with various analogs of pantothenic acid, when the non-nitrogenous portion is varied by condensing different hydroxy acids with  $\beta$ -alanine. Only very slight activity is found with any of these compounds, showing that even slight modifications of the molecular structure very greatly decrease the biological activity. The same general picture is found with higher animals for which pantothenic acid has metabolic importance.

With hydroxypantothenic acid it was observed (248) that the activity of this substance differed with different test organisms and with the mode of testing. In many cases growth curves could be obtained showing high activity (up to about 20% of that of pantothenic acid) at low dosage levels, with progressively lower activity at increasing concentrations. This phenomenon is unusual but appears to be an example of an inhibition due to the analog's blocking, when in sufficient concentration, some metabolic reaction in which pantothenic acid itself would normally be concerned.

#### 5. Pantothenic Acid Analogs as Growth Inhibitors

These observations of growth inhibitions with higher concentrations of hydroxypantothenic acid, where lower concentrations promote growth, connect with the question of deliberate modelling of inhibitory growth factor analogs. In the case of pantothenic acid this was first carried out by Snell (351, 352) who prepared pantoyl-taurine, *i.e.*, *N*-( $\alpha,\gamma$ -dihydroxy- $\beta,\beta$ -dimethyl-butyryl)-taurine, in which an  $\text{SO}_3\text{H}$  group is substituted for the

—COOH group of the  $\beta$ -alanine portion of pantothenic acid. McIlwain (235, 236, 238) independently made and tested the same and numerous other analogs. This is not the place for more than a brief mention of the extensive examination which is now being made of growth inhibition by analogs of pantothenic acid and other growth factors. In general it may be said that the findings show that those organisms which require pantothenic acid as an essential nutrient are susceptible to inhibition by analogs of pantothenic

TABLE XIV  
*Specificity of Pantothenic Acid*

Pantothenic acid analog (Non-nitrogenous acid moiety, all joined to $\beta$ -alanine)	Test organism	Activity per cent	References
HO·CH <sub>2</sub> ·C(CH <sub>3</sub> ) <sub>2</sub> ·CH(OH)·CO-:			
(pantoyl-) <i>dextro</i>	See Table XII	100	Table XII
“ <i>laevo</i>	Lactic acid bac- teria	0	373
HO·CH <sub>2</sub> ·C(CH <sub>3</sub> ) (CH <sub>2</sub> OH)·CH (OH)·CO- (hydroxypantoyl-)	<i>Sacch. cerevisiae</i> and lactic acid bacteria	1 to 25 (varies with test or- gan- ism)	248
HO·CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·CH(OH)·CO- ( $\alpha$ , $\delta$ -dihydroxyvaleryl-)	<i>Strept. hemolyt- icus</i> . Dochez NY 5	Slight	
	<i>Strept. hemolyt- icus</i> H-6905	“	449
HO·CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·CH <sub>2</sub> ·CH(OH)· CO-( $\alpha$ , $\epsilon$ , dihydroxy caproyl-)	<i>Strept. epidemi- cus</i> X 40	0.1	448
CH <sub>2</sub> ·CH(OH)·CH <sub>2</sub> ·CH(OH)·CO-		0.5	247
HO·CH <sub>2</sub> ·CH <sub>2</sub> ·C(OH)(CH <sub>3</sub> )· CH(OH)CO-	<i>Streptococcus</i>	0.5	
HO·CH <sub>2</sub> ·CH(CH <sub>3</sub> )·CH(OH)·CO-	<i>lactis</i>	0.5	
HO·CH <sub>2</sub> ·CH(OH)·CH(OH)·CO-		0	
HO·CH <sub>2</sub> ·CH <sub>2</sub> ·CH(OH)·CO-		0	
Pantothenic acid diphosphate	Lactic acid bac- teria	0	440

acid. The mode of action appears to involve the blocking of enzyme systems using pantothenic acid which are essential for the life of the organisms. However, to consider these growth inhibitions as due only to competitive or other inhibitions of enzyme systems normally using the essential metabolite, is probably too simple, as detailed analysis shows. Questions of different varieties of inhibitions, alternative reaction chains, adaptability of the organism, functional use of analogs, etc., all complicate the picture.

### 6. Other Organisms Using Pantothenic Acid

The fundamental role of pantothenic acid in metabolic processes of great generality in different forms of living matter is supported by the increasing number of observations of growth stimulation or nutritional requirement.

a) *Yeasts*. The discovery of pantothenic acid was originally made in connexion with yeast growth and has been reviewed by Williams (423). Perhaps more with yeast than with any other type of organism it must be stressed that the growth response to pantothenic acid is different for different strains. Different strains have different synthetic abilities for the various growth factors, and their synthetic abilities themselves are subject to change. The response to pantothenic acid is therefore much affected by the conditions of the experiment, including the behavior of the actual cells used for inoculation. Thus *Torula cremoris* examined by Koser and Wright (171) required pantothenic acid for prompt and abundant growth, although under some conditions slower growth took place without added pantothenic acid; the growth response depended to some extent on the composition of the basal medium and upon the presence of nicotinamide and biotin.

b) *Lower Fungi*. An X-ray induced mutant of *Neurospora crassa* required pantothenic acid for growth, the mutant having lost the power to synthesize it (12).

c) *Insects, etc.* Pantothenic acid has been found important in insect nutrition: mosquito larvae (377); *Plasmodium lophurae* (389); *Tribolium confusum*, *Plinus tectus*, *Silvanus surinamensis*, *Lasioderma serricorne*, *Sitodrepa panicea* (98, 99).

### 7. The Metabolic Function of Pantothenic Acid

At present little is known about the biological function of pantothenic acid. Those organisms which cannot synthesize it provide suitable material for investigating its role, since 'deficient' organisms can be easily obtained for study of its metabolic effects. A beginning has been made by Dorfman, Berkman, and Koser (67) and by Hills (128) using pantothenic-acid-deficient cells of *Proteus morganii*. It appears that pantothenic acid may be a component of a pyruvate oxidase system. McIlwain (241) has recently made a detailed analysis of the effects of pantothenate and pantoyltaurine upon the metabolism and growth of *Streptococcus hemolyticus*.

## VII. BIOTIN

### 1. Convergence of Nutritional Studies

The history of the discovery and the determination of structure of biotin has been well documented recently (130, 396, 398) and need not be repeated here except to connect up with the topic of the review. Attention should,

however, be drawn to the way in which at first apparently independent lines of research came together when it was found that biotin was the biologically active substance involved in all the phases of this work, which concerned such diversified biological types as bacteria, yeasts, and higher animals. That this should have been so was a consequence of the fundamental unity of all biochemistry. As with many other known essential metabolites biotin was discovered through the effects of a nutritional deficiency.

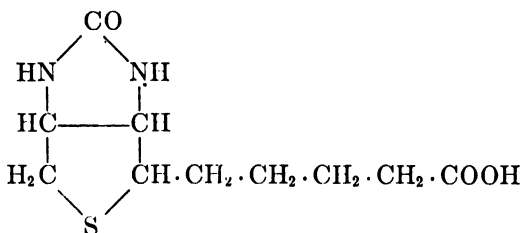
The observations of Wildiers (420) on the growth requirements of yeast, when small inocula were used for propagation in media of simplified composition, initiated the study of the growth requirements of yeasts in terms of the chemical composition of the medium needed for optimal growth. Thereafter, methods of study and an orientation of viewpoint were gradually evolved which resulted in a clear conception of the problems of yeast nutrition, which were seen to be no different in kind from those of other organisms (423).

Biotin was discovered and isolated by Kögl and Tönnis (160) using a yeast strain, "Rasse M," and an increased rate of growth under defined conditions as the biological test. Biotin was shown to be very widely distributed in biological materials of all kinds, including plant and animal tissues. Thus another part of the yeast "bios" complex, already separated into several distinct fractions, was identified.

During the same period as Kögl and Tönnis' work on yeast growth which led to the isolation of biotin, work was proceeding on the growth requirements of *Rhizobia*, the root-nodule nitrogen-fixing bacteria. Allison, Hoover, and Burk (3) found evidence for a highly active substance, which was not only specific but essential for the respiration of various strains of these organisms. Allison and Hoover (2a) showed that these organisms were unable to grow in carefully purified nutrient media of known chemical composition in the absence of this substance, which was named 'coenzyme R'. This substance was found to be widely distributed in natural sources and was, moreover, readily synthesized by *Azotobacter*. Biotin having been isolated it became possible to compare its properties with those of coenzyme R. Direct tests and much collateral evidence showed that the active substances of the two preparations were probably identical, both Nilsson, Bjälfve, and Burström (272) and West and Wilson (410, 411) showing that purified biotin preparations could supply the coenzyme R requirements of *Rhizobia*.

The third line involving biotin was the study of animal nutrition, first with rats, later extended to other animals. Here characteristic symptoms of "egg-white injury" were observed by Boas (27) in rats fed a diet containing egg-white as the source of protein. It was found that the animals could

be protected against the egg-white injury by some factor in potato starch, and a "protective factor—X" was postulated. Its distribution was studied and relatively large amounts were found in raw potatoes, yeast, milk, and liver. The subsequent history of the further characterization of the "protective factor X," the anti-egg-white injury factor, has been summarized by du Vigneaud (398). The attack on its chemical nature, and the clear recognition that the effect was due to a nutritional deficiency, induced by the egg-white protein and remediable by the protective factor (which was therefore in the class of vitamins) was at first carried out largely by P. György, who named the protective substance vitamin H. Later in association with du Vigneaud and co-workers the chemical work was intensified and it was eventually suspected that vitamin H might be biotin. Tests with vitamin H preparations showed intense biotin activity (for yeast) and co-enzyme R activity (for *Rhizobia*). Proof of the identity of biotin, co-enzyme R, and vitamin H followed from a comparison using an authentic specimen of Kögl's biotin methyl ester (115, 116, 396, 399). With the identification of vitamin H as biotin, new sources of material became available for the determination of its chemical structure, which is now known to be:



The chemical work has been reviewed by Hofmann (130) and Melville (245a). For present purposes the effects of biotin in the nutrition of higher animals will be omitted and attention confined to micro-organisms.

## 2. Yeasts

Following the initial work of Kögl and Tönnis (160) the stimulatory effects of biotin on the growth of many different strains of yeast have been intensively studied and different methods of observing the activity of the substance have been developed. Snell, Eakin, and Williams (354), using a different strain of yeast from that used by Kögl and Tönnis ("Rasse M") found that its sensitivity to biotin was increased if the concentration of other nutrilites was so adjusted that the biotin concentration became the limiting factor in growth. Williams, Eakin, and Snell (425) showed that three different strains of yeast were able to grow continuously from small inocula in the absence of added biotin; under appropriate conditions a striking response to added biotin was observed. This was because, without

added biotin under the conditions employed, the rate of biotin synthesis was limiting the rate of growth; hence the stimulation by an external source of supply.

In general it would seem that most yeasts can synthesize biotin to some extent. Whether or not a stimulation is observed on adding biotin to the culture medium depends on the actual rate of synthesis. This rate is determined by at least two inter-related sets of conditions: (a) the synthesizing abilities of the cells used, (b) the composition of the medium. Yeasts are able relatively easily to change their powers of synthesizing biotin. Leonian and Lilly (200) trained a yeast to grow readily on a medium of defined chemical composition without added nutrilites and found that the known yeast nutrilites, including biotin, were synthesized. Williams, Eakin, and Snell (425) studied the inter-relations of five yeast nutrilites: thiamin, biotin, inositol, pantothenic acid, and pyridoxin, in the growth of three strains of yeast. They observed phenomena which are of great interest to all studies of metabolic requirements, and are consistent with the view that nutritional requirements reflect the relative abilities of the cells in question to synthesize essential metabolites. Since all the synthetic processes of the cell are undoubtedly closely interwoven it is not unexpected that one given essential metabolite should affect the requirements for another. Supplementary effects were observed, the amount of growth obtained in a given time increasing as the external source of supply of the known nutrilites was made qualitatively richer. These are effects which might be expected when the rates of synthesis of several essential metabolites are sub-optimal. Growth limited by suboptimal rates of synthesis could thus be improved step by step by adding the known nutrilites one by one. For strains "Gebrüder Mayer" and "old process" yeast, omission of biotin from the medium seriously impaired growth, while for a strain from Fleischmann baker's yeast, omission of biotin was less serious, although a marked stimulation was seen on its addition. Certain replaceability effects were observed. With the Fleischmann strain, adding biotin alone or pantothenic acid alone increased the growth above that on the basal medium. But pantothenic acid showed its effect in less than 24 hours while biotin showed its effect first at 36 hours, with thereafter more rapid growth than with pantothenic acid alone. It thus appeared that in the presence of biotin the yeast developed the ability to synthesize pantothenic acid relatively rapidly, whereas in the presence of pantothenic acid alone the ability to synthesize biotin more rapidly was not developed. Without biotin, but in the presence of the 4 essential metabolites, inositol, thiamin, pyridoxin, and pantothenic acid, growth was still more rapid than with biotin alone, or with biotin + pantothenic acid, suggesting that biotin synthesis was facilitated by the presence of the other essential metabolites.



A similar effect with biotin alone was noted with the "old process" strain; practically no multiplication was observed until after 72 hours, when continued rapid growth began. In this case the delay in growth appeared to be due mainly to the slowness with which the organism became able to synthesize thiamin at an adequate rate. For the "Gebrüder Mayer" strain, pantothenic acid appeared indispensable for continued growth and biotin could neither replace it, nor, under the given conditions, facilitate the ability to synthesize it.

Leonian and Lilly (201) compared the relative requirements of 10 different strains of yeast for biotin, thiamin, inositol, pantothenic acid, and pyridoxin, using 72 hour growth. No strain grew significantly without biotin, all were restricted without pantothenic acid, one was dependent upon thiamin, etc. Again inter-relations in growth effect were observed and, in general, it could not be said that the nutritional need for any one substance was absolute; specific requirements depended on the presence of the other substances.

### 3. Bacteria

a) *General.* Biotin is now known to be important in the metabolism of many species of bacteria; many of these are listed in Table XV. For many bacteria biotin appears to be essential; there are many examples where lack of biotin prohibits growth entirely. If these organisms synthesize biotin at all it is at such a slow rate as to be ineffective, and in general they appear to be less able than the yeasts to be trained to synthesize biotin effectively. On the other hand there are many bacteria which can undoubtedly synthesize biotin readily (Table XV,C) and a few cases have been recorded where synthesis is evidently slow enough for a stimulation to be seen on adding biotin (Table XV,B)

Strictly it is incorrect to make a mechanical separation of bacterial species or strains into those for which biotin is *essential* as a nutrient and those for which it is a growth stimulant. Very limited rate and amount of growth may occur (see below) in a biotin deficient medium when a very slow rate of synthesis is possible. Depending on the condition of test this amount of growth may or may not be considered significant. But generally, in the observations recorded in the literature, a speed and massiveness of growth approaching that obtained in classic bacteriological media is the test of nutritional adequacy. When an organism responds in this degree to the addition of biotin (or other essential metabolite) to a deficient medium, then this metabolite can for certain purposes of classification be grouped as an "essential nutrient". There appear to be large enough quantitative differences in rate of synthesis to permit, for convenience, a grouping of those organisms for which an external source of biotin is needed

## TABLE XV

*Relations of Bacteria to Biotin**A. Bacteria for which Biotin is an important or essential nutrient*

	References
<i>Rhizobia:</i>	
<i>Bact. radicola</i> IV ( <i>Rh. leguminosarum</i> )	25, 272
<i>Rh. trifolii</i> , 205 and 209	411, 433
<i>Rh. meliloti</i> , 131	
<i>Rh. leguminosarum</i> , 311	
(other strains synthesize)	
<i>Clostridia:</i>	
<i>Cl. butylicum</i>	181, 183, 284, 365
<i>Cl. acetobutylicum</i>	180, 183, 276, 406
<i>Cl. tetani</i>	180, 267, 268
<i>Cl. sporogenes</i>	183, 193, 284
<i>Cl. felsineum</i> (Carbone)	183
<i>Lactobacillus arabinosus</i> 17-5	366
<i>Lactobacillus casei</i>	136, 190, 250, 345
<i>Streptococcus lactis</i> R	356
<i>Streptococcus hemolyticus</i>	134
a Group A strain C 2035	
<i>Staphylococcus aureus</i> (some strains; others synthesize)	287, 288
<i>Brucella:</i>	
<i>Br. abortus</i>	166, 170, 227, 228
<i>Br. melitensis</i>	228, 229
<i>Br. suis</i>	

*B. Bacteria for which Biotin has a stimulatory effect and which may synthesize it slowly*

<i>Rh. leguminosarum</i> H X (and other strains)	433
<i>Proteus vulgaris</i> (some strains)	49
<i>Staphylococcus aureus</i> (some strains)	162, 287, 288

*C. Bacteria which synthesize Biotin easily and show little if any stimulation by added Biotin*

Some strains of <i>Rhizobia</i>	4
<i>Mycobact. tuberculosis</i>	188, 189
<i>B. subtilis</i>	188, 189
<i>Aerobacter aerogenes</i>	49, 188, 189, 386
<i>Eberth. typhosa</i>	188, 189
<i>Proteus vulgaris</i>	49, 188, 189, 386
<i>Esch. coli</i>	188, 189
<i>Phytomonas tumefaciens</i>	244
<i>Serratia marcescens</i>	188, 189, 386
<i>Azotobacter vinelandii</i>	188, 189
<i>Alkaligenes fecalis</i>	49, 188, 189
<i>B. anthracis</i>	188, 189
<i>Pseudomonas aeruginosa</i>	188, 189
<i>Pseudomonas fluorescens</i>	386

for good normal growth. These are included in Table XV, A. Comments on some special cases follow.

b) *Rhizobia*. The original observations on co-enzyme R, later identified as biotin, were made by Allison, Hoover, and Burk (3) who reported a highly active substance present in a wide range of biological materials which markedly increased the rate of respiration of *Rhizobium trifolii* cells, to  $Q_{O_2} = 1000$ , and also of the root nodule organisms of alfalfa, pea, and bean; respiration stimulation was also observed with other bacteria. The co-enzyme R was found to be synthesized in large amount by the free living nitrogen-fixer *Azotobacter vinelandii*, an organism having "substantially the highest rate of respiration ( $Q_{O_2} = 5000$ ) possessed by any organism." It was recorded briefly that legume nodule bacteria could grow as well on a chemically defined medium containing highly active preparations of the respiration factor, as when the latter was replaced by yeast extract. The nutritional problem was studied in greater detail by Allison and Hoover (2a) who attempted to isolate co-enzyme R. They showed that strains of *Rh. trifolii*, *Rh. leguminosarum*, and *Rh. meliloti* were unable to grow on a chemically defined medium without addition of co-enzyme R. On the same medium the free-living *Azotobacter vinelandii* grew well without added co-enzyme R. Allison, Hoover, and Burk (3) pointed out that in legume symbiosis the host plant probably furnished the root-nodule *Rhizobia* with the essential co-enzyme R, which the free-living *Azotobacter* could synthesize for themselves. The observations of West (407) on the different nutrient requirements of bacteria within and without the rhizosphere are of interest in this connexion (see p. 121). At this stage the work rested until biotin had been identified with co-enzyme R. Wilson and Wilson (433) have made a careful study of the role of biotin in the growth of *Rhizobia*. They found that it was possible to obtain continuous growth in serial transfer of *Rhizobium trifolii* 205 and *Rhizobium meliloti* 131 in media and under cultural conditions where rigorous precautions had been taken to exclude biotin. Growth was independent of the concentration of glucose or sucrose or the number of transfers. Unless specially purified, as these specimens were, glucose and sucrose were found to be contaminated with traces of biotin. Continuous transfer of these organisms, and of *R. leguminosarum* 311 and *R. trifolii* 209, without decrease in growth was obtained in a purified medium containing synthetic glycerol as carbon source. But in all these continuous transfer experiments on purified media the maximum growth was only about  $\frac{1}{10}$  that obtained with a similar medium to which the optimum amount of biotin was added. The organisms obtained with *R. trifolii* 205 after continuous transfer in the purified biotin-deficient media were apparently normal, *i.e.*, the ability to infect and fix nitrogen in association with the clover plant and the respiration activity in glucose, measured by

the  $Q_{O_2}$  (N), were unchanged. Direct attempts to demonstrate synthesis of biotin by these organisms were unsuccessful, but an amount of biotin may have been present which was below the limits of detection by the method used. During continuous transfer for over a year on purified synthetic media without biotin, *R. trifolii* 205 did not vary markedly in rate or extent of growth. Wilson and Wilson suggested two possibilities to explain the continued sub-maximal growth on biotin-deficient media: (a) the organism synthesized biotin to a very limited extent, so that the biotin available remained a limiting factor for growth while an excess of other nutrients remained unused; (b) growth and metabolism proceed at a greatly reduced level in the complete absence of biotin. Explanation (b) implies that the organism can use, although much less effectively, metabolic reactions for the building-up of its substance which do not involve biotin. It was found that one strain, *R. leguminosarum* HX, which was not stimulated by added biotin, did in fact synthesize this substance. Allison and Minor (4) also showed that some strains of *Rhizobia* synthesized 'co-enzyme R' and excreted it into the medium. Wilson and Wilson concluded that *Rhizobia* could be divided into three groups on the basis of their biotin requirements:

- (1) A few strains were unable to grow at all in a biotin-deficient medium.
  - (2) In a biotin-deficient medium the majority of strains examined grew very poorly and reached a population only about one tenth of that possible when biotin was added. But continuous serial transfer in the biotin-deficient medium was possible. These strains responded markedly to the addition of biotin.
  - (3) A few strains attained practically maximum growth in the biotin-deficient medium. One such strain was shown to synthesize biotin.
- Explanation (b) above may of course explain the behavior of group 2, but the whole picture is more consistent with explanation (a) in which the nutritional requirements reflect an increasing power to synthesize biotin in proceeding from group 1 to group 3.

Comparisons of the ability of different strains of the same species to synthesize biotin have been made with other organisms besides the *Rhizobia*, and similar differences are implicit from records in the literature.

c) *Staphylococcus aureus*. Certain strains of *Staph. aureus* were grown in media of known composition, and the essential metabolites needed to be given were thiamin and nicotinic acid (104, 156). Continued serial sub-culture in this medium was possible giving a reasonable amount of cells. Nevertheless Kögl and van Wagtenonk (162) showed that a strain which grew moderately on a basal medium, containing nicotinic acid and thiamin, was much stimulated by added biotin (0.005  $\mu\text{g./ml.}$ ) the mass of growth at 48 hours being increased 7 or 8 times. Inter-relations of the effects of the 3 growth factors were observed; about 100 times less thiamin and nicotinic

acid were required in the presence of biotin to give the same mass of growth as was given by thiamin and nicotinic acid without biotin. Porter and Pelczar (287, 288) carefully studied the growth requirements of some 17 representative strains of *Staph. aureus*. They found that certain strains were unable to grow in continued sub-culture on a medium of defined chemical composition including nicotinic acid and thiamin, and required to be given biotin before growth could occur; other strains which grew in the medium without biotin were not stimulated by adding it. The general picture with *Staph. aureus* therefore appears similar to that with *Rhizobia*, some strains requiring biotin as a component of the nutrient medium while others do not, presumably because they can synthesize biotin more or less rapidly. Landy and Dicken (188) found that biotin was synthesized by the strain of *Staph. aureus* they examined.

#### 4. Bacterial Synthesis of Biotin

The general picture of the role of biotin in bacterial growth is that of an essential metabolite required for use in some common and fundamental growth processes, and those organisms which cannot synthesize it readily enough are more or less dependent on an external source of supply. Thompson (386) studied quantitatively biotin synthesis by species of bacteria which could grow in a biotin-deficient medium. *Serratia marcescens*, *Proteus vulgaris*, *Aerobacter aerogenes*, and *Pseudomonas fluorescens* synthesized about 2.5 to 7.0  $\mu\text{g}$ . biotin/g. dry cells; a much larger quantity was excreted into the medium. There were indications that while the synthetic powers of the different species varied as to total amount produced, the amount of biotin retained within the cells was relatively constant. The liberation of biotin during growth ran closely parallel to amount of growth, indicating that the biotin excreted came from an overproduction, rather than from cell autolysis. Growth of the cells in media containing added single growth factors (riboflavin, nicotinic acid, pantothenic acid, etc., which these organisms could synthesize) did not cause any detectable change in the amount of biotin synthesized, Table XV, C.

#### 5. Lower Fungi, etc.

In addition to the higher animals, bacteria, and yeasts already mentioned, effects have been observed with many different species of lower fungi. Kögl and Fries (161) first showed that biotin was important in the nutrition of lower fungi when it was shown that it was one of the growth factors required by *Ashbya (Nematospora) gossypii* (50, 51). Many other lower fungi are now known to require biotin as a nutrient or to be stimulated in growth, and others have been shown to synthesize biotin (Table XVI). *Insects*. Biotin has been found important in the nutrition of *Tribolium confusum* (97, 98, 99).

## 6. Specificity

Little direct work on the biological specificity of biotin using analogs and derivatives has yet been done. During the determination of the struc-

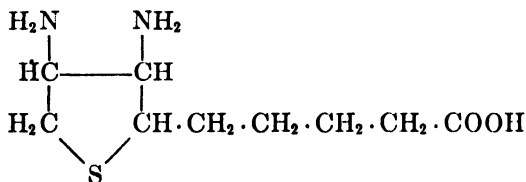
TABLE XVI  
*Biotin and the Growth of Lower Fungi*

Biotin as nutrient	References	Biotin not required as nutrient and probably synthesized	References
<i>Ashbya gossypii</i>	161, 313		
<i>Melanospora destruens</i>	125	<i>Morrellia albicans</i>	189
<i>Mitrula paludosa</i> (spore ger- mination)	103	<i>Aspergillus niger</i>	
<i>Marasmius androsaceus</i>	205	<i>Aspergillus oryzae</i>	
<i>Ophiobolus graminis</i>	415	<i>Epiphyton interdigitale</i>	
<i>Neurospora</i> (5 races)	52	a <i>Penicillium</i>	
<i>Diplodia macrospora</i>	370	a <i>Mucor</i>	199
<i>Ophiostoma</i> ( <i>Ceratostomella</i> )	103	<i>Mucor ramannianus</i>	
<i>pini</i>		<i>Rhizopus suinus</i>	
<i>Ceratostomella rudicicola</i>	318	<i>Fusarium nivium</i>	
		<i>Fusarium avenaceum</i>	
		<i>Phytophthora erythroseptica</i>	
		<i>Phytophthora gonapoides</i>	

Biotin stimulates growth:

<i>Ophiostoma</i> ( <i>Ceratostomella</i> ):	103
<i>O. stenocerus</i>	
<i>O. coeruleum</i>	
<i>O. quercus</i>	
<i>O. fagi</i>	
<i>O. piliferum</i>	
<i>Ceratostomella leptographioides</i>	318

ture of biotin, du Vigneaud, *et al.* (400) obtained by barium hydroxide hydrolysis the diaminocarboxylic acid:



by opening of the urea ring of biotin. This acid was found to possess about 10% of the activity of biotin itself in the assay using yeast growth stimulation (356). It is possible that this degradation product of biotin can perform to some extent the function of biotin in metabolism; experiments such as the respiration stimulation of non-multiplying *Rhizobia* cells might test this point. But an explanation more consistent with the "essential metabolite" function of biotin would appear to be that the diaminocarboxylic

acid served as a rather inefficient building-stone for biotin synthesis by the yeast (see p. 170). For most organisms (yeasts, bacteria, lower fungi) the biotin methyl ester is as effective as the free acid, but Shull, Hutchings, and Peterson (345) found that for their *Lactobacillus casei* strain only the free acid could be used and not the methyl ester, which the organism was unable to hydrolyze. For further discussion see the section added in proof, page 228a.

### 7. Pimelic acid

Pimelic acid,  $\text{HOOC} \cdot (\text{CH}_2)_5 \cdot \text{COOH}$ , was discovered as a bacterial growth factor by Mueller (257, 258, 259, 262) for certain strains of *C. diphtheriae*:—the "Allen" strain and some Park 8 strains. This was the first time any specific physiological function had been observed with this substance. Mueller showed that other dicarboxylic acids of the same homologous series could not be used in place of pimelic acid. At present no other bacteria have been observed to require pimelic acid, and many strains even of *C. diphtheriae* do not need it as a nutrient. A possibility that the activity of pimelic acid might be due to its serving as a precursor in the biosynthesis of biotin was advanced by du Vigneaud, *et al.* (401). Examination of the structural formula of biotin shows that pimelic acid could supply the fatty-acid side chain and contribute a further 2 C atoms to the ring. To test this hypothesis biotin was substituted for pimelic acid in a nutrient medium for the "Allen" strain of *C. diphtheriae*, which normally requires pimelic acid. It was found that maximum growth was obtained with 0.15  $\mu\text{g.}/\text{ml.}$  medium, of either substance; at lower concentrations biotin was more effective than pimelic acid:

Biotin $\mu\text{g.}/\text{ml.}$	Pimelic acid $\mu\text{g.}/\text{ml.}$	Bacterial N $\text{mg.}/10 \text{ ml. medium}^*$
0.0005	0.002	1
0.0015	0.0042	2
0.0035	0.0075	2.5

\* 64 hr. cultures, 34–35°.

This is the clearest evidence there is at present to account for the effect of pimelic acid as a bacterial essential nutrient. The particular strains of *C. diphtheriae* which require pimelic acid as a nutrient are probably strains lacking the ability to synthesize biotin. And this defective synthetic power would appear to concern the fatty acid side chain of biotin, since when pimelic acid is provided, the rest of the molecule appears to be synthesized. This is supported by the finding that *C. diphtheriae* "Allen" strain, using pimelic acid, was not inhibited by the antibiotin factor avidin, whereas inhibition did occur when biotin was the external source of growth factor (see next section). (Cf. also 73a.)

### 8. The Antibiotin Factor and Bacterial Metabolism

Identification of biotin as vitamin H, the anti-egg-white-injury factor, and the demonstration that the vitamin H deficiency was caused by something in egg-white has already been noted. Eakin, Snell, and Williams (75, 76) prepared concentrates from egg-white which could inhibit the action of biotin *in vitro*, in the yeast assay, similar preparations being equally active in producing egg-white injury in rats. The antibiotin factor was found to be a protein having some characteristics of an albumin. This active protein (for which the name *Avidin* was proposed) was shown to be able to combine stoichiometrically with biotin. Woolley and Longsworth (450) independently prepared the antibiotin factor from egg-white, and obtained preparations which were homogeneous on electrophoresis and in the ultracentrifuge. The data regarding molecular size indicated that the antibiotin factor had a molecular weight of about 70,000, and that it combined with biotin in a molecular ratio of 1:1. Woolley and Longsworth showed that the antibiotin factor inhibited the growth of *Cl. butylicum*, which required biotin as a nutrient component.

Numerous interesting consequences follow from the discovery of a naturally-occurring growth inhibitor such as Avidin,<sup>1</sup> which throw an entirely new light on the problems of nutritional sufficiency.

Growth inhibition by the antibiotin factor has been shown with several micro-organisms which need an external source of biotin for growth: Yeast (Fleischmann strain) (75, 76); *Cl. butylicum* (193, 450); *Lactobacillus casei*; *Lactobacillus arabinosus* 17-5; *Streptococcus lactis* R; *Staphylococcus aureus* X3, Y; *Cl. acetobutylicum* A-211; *C. diphtheriae* "Allen" strain (193); this inhibition could be reversed by adding excess biotin. Landy, Dicken, Bicking, and Mitchell (193) have made an extremely interesting use of the antibiotin factor to discover bacteria which require an external source of biotin, in those cases where the organism has not yet been grown in chemically-defined media. Having shown that many organisms, already known to require an external source of biotin, were inhibited in growth by antibiotin factor (see above) and that organisms known to synthesize biotin (188) were not inhibited by the antibiotin factor, organisms whose biotin requirement was unknown were examined for inhibition by the antibiotin factor. Several organisms were found which were thus inhibited, and this inhibition could be completely reversed by excess of biotin. These or-

<sup>1</sup> There is a good deal to be said for preferring to name the egg-white-injury factor the antibiotin factor, as proposed by Woolley and Longsworth, rather than Avidin, since the former name relates it to the substance, biotin, with which it unites in producing a nutritional deficiency; furthermore there is evidence that there may be a class of these antivitamin substances existing in nature. Woolley (444) has already indicated the existence of an anti-thiamin factor. A uniform nomenclature would appear to be a convenience.



ganisms were: *Cl. chauwei*, *Cl. botulinum*, *Cl. welchii*, *Cl. histolyticum*, *Cl. sporogenes*, *Diplococcus pneumoniae*, *Lactobacillus casei*, *L. acidophilus*, and *Bacillus brevis*. That *Cl. sporogenes* required an external source of biotin had already been briefly reported by Peterson, McDaniel, and McCoy (284). On the basis of these findings, Landy, *et al.* (193) predicted that this latter group of organisms would in fact be found to require an external source of biotin for growth. The finding that *Lactobacillus casei* required biotin (190) was obtained as a test of this hypothesis. It remains to be shown directly that the other organisms inhibited by the antibiotin factor do in fact require biotin as a nutrient. It seems probable that this will be found to be the case.

The use of specific inhibitors operative against essential metabolites opens a new route of attack for the discovery of the external growth factor requirements of bacteria and other organisms. Once an essential metabolite is known it may be possible deliberately to design specific growth inhibitors (90) or to find naturally-occurring ones, like the antibiotin factor; these specific inhibitors will then enable a rapid survey of bacteria to be made to reveal their requirements for the specific essential metabolite.

Since the antibiotin factor does not inhibit those bacteria which synthesize biotin, the antibiotin factor appears to combine only with the externally supplied biotin and cannot penetrate effectively to the sites of biotin synthesis and utilization. This is analogous to the findings with the synthetic inhibitory growth factor analogs (*e.g.*, pyrithiamin and thiamin, pantooyl taurine and pantothenic acid, pyridine-2-sulfonamide and nicotinamide) where the inhibitory action is largely confined to those organisms which do not synthesize the growth factor.

In conformity with this is the observation of du Vigneaud, *et al.* (401) that the "Allen" strain of *C. diphtheriae* was inhibited by the antibiotin factor when using an external source of biotin, but not when using pimelic acid. In the latter case it seems probable that pimelic acid was used as a precursor in the biosynthesis of biotin, *i.e.*, that biotin was synthesized out of the range of action of the antibiotin factor. This observation of the different effects of antibiotin itself supports the view that pimelic acid is a biotin precursor. Similarly du Vigneaud, *et al.* (400) found that the activity for yeast of the diaminodicarboxylic acid derived from biotin by opening the urea ring was not inhibited by the antibiotin factor. This showed, not only that the lowered activity (10% of biotin activity on a molecular basis) of the diaminodicarboxylic acid was not due to unchanged biotin, but that the urea ring of biotin was concerned in the combination of antibiotin with biotin. Failure of antibiotin to inhibit the growth-promoting effect of the diaminodicarboxylic acid would be in conformity with the view that the latter was used as a biotin precursor in biosynthesis.

VIII. *p*-AMINO BENZOIC ACID1. *Discovery of Biological Importance*

The essential metabolite *p*-aminobenzoic acid is the first substance of this class which has been discovered not directly by observation of a nutritional deficiency, but indirectly as an antagonist of a growth-inhibitory substance. The sulfanilyl anti-bacterial drugs, against which *p*-aminobenzoic acid shows its antagonistic effect, were evolved empirically without the deliberate design of blocking any known physiological function in the organisms to be attacked. The present case may serve as an example of another means of studying the biosynthetic net-work of micro-organisms, namely by finding natural antagonists of growth inhibitors.

It is worth noting some points about the discovery of the metabolic importance of *p*-aminobenzoic acid because they illustrate clearly one of the main theses of this review. When the Dept. of Bacterial Chemistry (directed by P. Fildes) of the (British) Medical Research Council was studying the exact nutrient requirements of hemolytic streptococci (242) sulfanilamide was being successfully used in the treatment of streptococcal infections, and the possibilities of the then new sulfonamide drugs were topical subjects for discussion. When, therefore, glutamine was found to be very important in the growth requirements of a highly virulent strain of streptococcus, the following possibility suggested itself to biochemists familiar with competitive inhibitions of enzyme reactions;—if the enzyme reaction concerned with the utilization of glutamine by the streptococci involves the amide group, then the amide group of sulfanilamide might enter into competition with that of glutamine. General conceptions of bacterial nutrition, which formed the theoretical background to the work of the laboratory (91a, 154), already emphasized that nutritional requirements reflect the ability to synthesize essential metabolites, the latter being substances which play essential parts in chains of syntheses necessary for growth (90). Hence an immediate connexion between growth inhibitors and interference with essential metabolic reactions was always implicit. The effect of sulfanilamide was therefore considered from this point of view. Independently Lockwood (208) and McIntosh and Whitby (243) had suggested, from indirect evidence, that the inhibitory action of sulfanilamide was due to inactivation of bacterial enzymes. Many unsuccessful attempts to show a reversal by glutamine of sulfanilamide inhibitions of streptococci forced the conclusion that glutamine was not the substance with the utilization of which the drug interfered. Other compounds of importance in bacterial growth also proved ineffective, but Woods (438) found that extracts of yeast could reverse the inhibition of streptococcal

growth by sulfanilamide. Working independently on a similar hypothesis Stamp (369) found that extracts of streptococci themselves could reverse the inhibition, and Green (109) obtained preparations from *Brucella abortus*. Thus natural antagonists of sulfanilamide were of wide natural distribution. Fractionation of yeast extracts and study of the chemical and other properties of the active substance suggested that it might be chemically related to sulfanilamide itself. A similar conclusion was reached from a study of the quantitative relationship of sulfanilamide and preparations of the active antagonist in growth tests. A constant quantitative relation between inhibitor and its antagonist was found which, according to Woods, "was reminiscent of the competitive inhibition of enzyme reactions by substances chemically related to the substrate or product. From this point

TABLE XVII

*Anti-Sulfanilamide Activity of Substances Related to p-Aminobenzoic Acid* (438).

Test organism: *Streptococcus hemolyticus* "Richards"

Conc. of sulfanilamide:  $3.03 \times 10^{-4}$  M

Substance	Active at conc. M
<i>p</i> -Aminobenzoic acid	$1.2-5.8 \times 10^{-8}$
<i>o</i> - " "	inactive
<i>m</i> - " "	$0.9 \times 10^{-8}$
<i>p</i> -Nitrobenzoic "	$1.8 \times 10^{-4}$
<i>p</i> -Acetaminobenzoic acid	$1.8 \times 10^{-4}$
Ethyl- <i>p</i> -aminobenzoate (benzocaine)	$3.6 \times 10^{-8}$
Diethylaminoethyl ester of <i>p</i> -aminobenzoic acid (novocaine)	$5.8 \times 10^{-8}$
<i>p</i> -Aminobenzamide	$1.4 \times 10^{-8}$
2-( <i>p</i> -Aminobenzylamino)-pyridine	$0.9 \times 10^{-8}$

*Inactive* were: *p*-hydroxybenzoic acid; *p*-toluic acid; benzoic acid; benzamide; *p*-aminophenol (inhibits growth down to  $3.5 \times 10^{-8}$  M); *p*-aminophenylarsenic acid; sulfanilic acid (inhibits at  $10^{-8}$  M).

of view the factor would be considered to be the substrate (or product) of the enzyme reaction in question and sulfanilamide the substance of related chemical structure inhibiting the reaction" (438). Testing of compounds structurally related to sulfanilamide (*p*-aminobenzene sulfonamide) was carried out, and *p*-aminobenzoic acid proved to have very high activity. This compound had not until then been known to have any notable biological activity. The specificity of *p*-aminobenzoic acid in abolishing sulfanilamide inhibition of streptococci is illustrated in Table XVII, from the original paper by Woods. Wyss (455) analyzed mathematically the effects of *p*-aminobenzoic acid and sulfanilamide on the growth rate of *Esch. coli* and concluded that the inhibition was of the competitive type. Woods' findings concerning specificity of *p*-aminobenzoic acid in reversing sulfanilamide (and sulfapyridine) inhibitions were obviously of cardinal

importance, and gave a new impetus to studies of bacteriostasis. While Woods did not succeed in isolating *p*-aminobenzoic acid from yeast, it was clear that in *p*-aminobenzoic acid a substance of great physiological importance had been recognized. The compound itself was later isolated from yeast by Blanchard (26), thus proving its occurrence in natural products.

The general conception of a rational approach to research in chemotherapy based on the objective of *interfering with essential metabolic reactions by means of inhibitors modelled on essential metabolites* was published by Fildes (89, 90) and Woods (438). The very great amount of work in this field of antibacterial chemotherapy, to which the discovery of the biological effects of *p*-aminobenzoic acid and the theory of its reversal of sulfanilyl antibacterial agents gave rise, cannot be discussed here. Some of it has been reviewed by Ansbacher in the previous volume (8a). It will be sufficient to point out that we are only at the beginning of understanding the function of *p*-aminobenzoic acid and of the mode of action of antibacterial agents of the anti-metabolite type. It is already clear that the simple theory is too simple and that in order to explain all the phenomena of inhibition and reversals, including those by substances other than *p*-aminobenzoic acid, the whole physiology of the attacked bacteria will need to be known in far greater detail than at present. One valuable avenue of approach to this will be the detailed study of nutrient requirements and of the inter-relations of nutrient components in promoting optimal growth. For the present this review must be confined to consideration of *p*-aminobenzoic acid as a growth factor and inhibitions will only be considered so far as they bear directly on this.

## 2. *p*-Aminobenzoic Acid as Growth Factor

On the basis of the conception that nutrient requirements reflect the organism's ability to synthesize its essential metabolites, Woods (438) predicted that *p*-aminobenzoic acid might be found to be an essential growth factor for some organisms, since sulfanilamide appeared to block a normal essential metabolic reaction for which *p*-aminobenzoic acid was required. This prediction was verified when it was found (323) that the substance was required as a growth factor by *Cl. acetobutylicum*, followed by similar findings with other organisms. The following is a list of organisms for which *p*-aminobenzoic acid is an essential growth factor: *Cl. acetobutylicum* (182, 183, 184, 278, 323); *Lactobacillus arabinosus* (139, 204); *Acetobacter suboxydans* (185, 192, 392); *C. diphtheriae gravis* type, "Dundee" sub-type (53); *Cl. butylicum* strain 28 (1 of 7) (183); *Cl. felsincum* No. 41 (183); *Neurospora* mutant "aminobenzoicless" (382, 388) Microbiological assay methods have been based on these findings. It will be noted that in addition to bacteria, a mold mutant requires *p*-aminobenzoic acid.

### 3. Specificity of *p*-Aminobenzoic Acid as Bacterial Growth Factor

This has been examined using *Acetobacter suboxydans* and *Cl. acetobutylicum*, and the results (Table XVIII) for these organisms are in substantial agreement. There are some discrepancies in the observations recorded for *Cl. acetobutylicum* with a few of the compounds. Rubbo and Gillespie (324) reported that *p*-aminophenylacetic acid was 10 times more active than *p*-aminobenzoic acid, but other observers have failed to confirm this (183, 456). Lampen and Peterson (183) found that the apparent activity of commercial *p*-aminophenylacetic acid fell from 0.1% of the potency of *p*-aminobenzoic acid to 0.002%, after two recrystallizations. Rubbo and Gillespie (324) and Landy and Wyeno (197) noted that *p*-aminophenylace-

TABLE XVIII  
Specificity of *p*-Aminobenzoic Acid as Bacterial Growth Factor

Compound	Organism		
	<i>Acetobacter suboxydans</i>	<i>Cl. acetobutylicum</i>	
	References: (192) % Activity compared with <i>p</i> -aminobenzoic acid	(183)	(324)
<i>p</i> -Aminobenzoic acid	100	100	100
<i>o</i> -Aminobenzoic acid	0	0	0
<i>m</i> -Aminobenzoic acid	0	0	0
<i>o</i> -Hydroxybenzoic acid	0	0	0
<i>p</i> -Aminobenzoic acid methyl ester	—	<0.01	—
“ “ “ ethyl “	0.06	<0.01	—
<i>p</i> -Aminobenzamide	—	—	<0.01
<i>N</i> -Acetyl- <i>p</i> -aminobenzoic acid	0	0.4	—
<i>N</i> -Benzoyl- <i>p</i> -aminobenzoic acid	—	<1.0	—
<i>p</i> -Aminobenzoyldiethylaminoethanol (procaine)	2.0	10-20	1.0
<i>p</i> -Glycylaminobenzoic acid	9.0	—	—
<i>p</i> -Aminophenylglycine	0	—	—
<i>p</i> -Nitrobenzoic acid	—	90-100	10
<i>p</i> -Aminobenzoic acid- <i>N</i> - <i>d</i> -ribose	—	90	—
“ “ “ “ - <i>N</i> - <i>l</i> -arabinoside	—	104	—

tic acid did not have anti-sulfanilamide activity, nor had the substance appreciable growth-promoting activity for *Acetobacter suboxydans* (183). On balance it would appear that the original observation of Rubbo and Gillespie requires re-investigation. The activity of “marfanil,” *p*-(amino-methyl)-benzene sulfonamide, which is not antagonized by *p*-aminobenzoic acid, opens a new aspect of bacteriostatic activity of compounds closely related to the latter compound. There are also considerable differences in the reported activity of *p*-nitrobenzoic acid. Generally, the specificity of the *p*-aminobenzoic acid structure is high; the lowered activity of derivatives appears related to the ability of the test organism to derive the master-compound from it. With substituted *p*-aminobenzoic acids, the 2-F com-

pound was about one-third as active as the parent compound; the 2-Br, 2-I, and 3-COOH derivatives had only traces of activity (456). The key role of the *p*-structure agrees with the general effectiveness of the corresponding sulfanilyl structures and the ineffectiveness of the *o*- and *m*-structures. The direct test of compounds related to *p*-aminobenzoic acid as growth factors gives results in good agreement with the ability of these compounds to reverse sulfanilamide inhibition

TABLE XIX

*Bacteriostatic Effects of Compounds Related to p-Aminobenzoic Acid (142)*

Compound	Minimum concentration ( $\mu\text{g./ml.}$ ) for bacteriostatic effect over 64 hr. period			Max. conc. tested
	<i>E. coli</i>	<i>S. hemolyticus</i>	<i>D. pneumoniae</i>	
Sulfanilamide	40	12	50	
Sulfapyridine	4	10	10	
Sulfathiazole	0.4	5	5	
Sulfadiazine	0.4	2	2	
3-Methyl-4-amino-benzoic acid	300	300	—	4,000
3-Bromo-4- " " "	600	600	—	2,000
2-Methyl-4- " " " HCl	4,000	—	—	8,000
2-Acetyl-amino-4-amino-benzoic acid	—	8,000	—	8,000
3-Methoxy-4- " " "	8,000	200	—	8,000
2-Chloro-4- " " "	200	—	—	2,000
3-Methyl-4-amino-benzamide	2,600	1,400	—	3,200
5-Nitrothiophene-2-carboxamide	4	5	40	500
5-Nitrothiophene-2-carboxylic acid	40	2.5	20	2,000
Methyl-2-(5-acetyl-amino-thienyl)-ketone	20	40	60	80
6-Amino-3-carboxy-pyridine	5	12	—	1,000

#### 4. Growth Inhibitors without Sulfonamide Groups Antagonized by *p*-Aminobenzoic Acid

If *p*-aminobenzoic acid is the key substance with which the sulfanilyl drugs compete, then substances modelled on the *p*-aminobenzoic acid structure, but not containing the sulfonamide group, might be able to produce bacteriostatic effects comparable with those of the sulfanilyl compounds. Johnson, Green, and Pauli (142) tested a number of such compounds. The test organisms used were *Escherichia coli*, *Streptococcus hemolyticus* (Group A), *Diplococcus pneumoniae* Type III. Twelve compounds showing bacteriostatic effects which could be reversed by *p*-aminobenzoic acid were found; nearly all were much less inhibitory than the sulfanilyl compounds sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine tested under the same conditions. Some of the results are collected in Table XIX, but the detailed tables of the original should be consulted. Among the active ring-substituted analogs the bacteriostatic potency

never exceeded one-fifth that of sulfanilamide or about 1/100th that of sulfathiazole or sulfadiazine. The bacteriostatic effect of inhibitory analogs was completely reversed by *p*-aminobenzoic acid, making probable that the inhibition was due to interference with the metabolic reactions in which *p*-aminobenzoic acid is normally used. Certain conclusions can be drawn from the observations of the activity or inactivity shown by the *p*-aminobenzoic acid analogs (for the compounds found inactive see original). Monosubstitution by neutral or weakly electropositive groups at positions 2 or 3 in *p*-aminobenzoic acid yields inhibitory compounds, but disubstitution at 2,3 or 3,5 gave non-inhibitory compounds. The 4-amino group could not be replaced, except by a nitro group, without loss of inhibitory activity. In the case of the 4-nitrobenzoic acid, which slightly inhibited *Esch. coli* at high concentrations, the inhibition was only transient, and similarly with *p*-aminobenzamide; it appeared that these could be rapidly changed by the organism to *p*-aminobenzoic acid, thus abolishing the inhibition. The high activity of the isostere analogs 5-nitrothiophene carboxamide and the corresponding carboxylic derivative, and of 6-amino-3-carboxypyridine, is very interesting; in contrast was the inactivity of the thiazole isostere, 2-amino-5-carboxythiazole and the furan isosteres, 5-nitro- and 5-acetyl-amino furoic acid. The active inhibitors were antagonized by *p*-aminobenzoic acid, *i.e.*, the inhibition was a true anti-*p*-aminobenzoic acid effect, and not due to inhibition of some other key metabolic reaction. The main conclusion which emerges is that the sulfonamide group or a substituted sulfonamide group, in place of the carboxyl group of *p*-aminobenzoic acid, is not the only way in which that molecule can be altered so that an active competitor can be made, although the  $\text{—SO}_2\text{NH}_2$  or  $\text{—SO}_2\text{NH—}$  groups are clearly highly efficient for this end. This may be related to the unphysiological character of the sulfonamide group itself which renders it impossible for the organisms to convert the sulfonamide to a usable structure, quite apart from the closeness of size and physical constants with which the  $\text{—SO}_2\text{NH—}$  group may simulate the natural metabolite as an enzyme competitor.

##### *5. Growth Inhibition by p-Aminobenzene Sulfonamide Derivatives as Evidence for Need of p-Aminobenzoic Acid in Metabolism*

If it be allowed that inhibition by sulfanilyl derivatives and the annulment of this inhibition by *p*-aminobenzoic acid, with a high degree of structural specificity, be an indication that the latter compound is of metabolic importance, then the list of organisms for which *p*-aminobenzoic acid may be eventually shown to be an essential metabolite may be greatly extended. It will be seen from the following list that the sulfanilyl-*p*-ami-

nobenzoic acid antagonism is demonstrable with the very widest variety of organisms:

Isolated tomato roots	(35)
Fungi: <i>Neurospora crassa</i> normal and "aminobenzoicless"	
mutant	(382)
<i>Trichophyton purpureum</i>	(62)
Diatoms: <i>Nitzschia palea</i>	(419)
Yeast	(191)
Virus: <i>Lymphogranuloma venereum</i>	(93, 131)

Numerous species of bacteria which do not require *p*-aminobenzoic acid as growth factor are inhibited by sulfanilyl compounds, the inhibition being reversed by *p*-aminobenzoic acid, *e.g.*, meningococci (385); *Esch. coli* (149); *Diplococcus pneumoniae* (197); *Staph. aureus* (197). In the two latter cases the specificity of *p*-aminobenzoic acid was examined; the *o*- and *m*-compounds were not active in abolishing the inhibition.

#### 6. Synthesis of *p*-Aminobenzoic Acid by Bacteria

Using *Acetobacter suboxydans* as test organism for *p*-aminobenzoic acid assay Landy, Larkum, and Oswald (194) showed that a large number of bacteria which grew without added *p*-aminobenzoic acid synthesized material which could supply the growth requirement of *Acetobacter suboxydans* for *p*-aminobenzoic acid. Short of actual isolation this test appears sufficient. It supplies good evidence for a general metabolic function of *p*-aminobenzoic acid. The sulfanilyl bacteriostatic compounds, as is well known, are not limited in their inhibition to organisms for which *p*-aminobenzoic acid is an essential growth factor, *i.e.*, organisms which do not synthesize it. In this character these bacteriostatic agents differ from some of the other growth inhibitors modelled on essential metabolites, which act preferentially against those organisms which must acquire the essential metabolite from an outside source because they do not synthesize it. Why this should be cannot yet be explained. The difference could depend upon the locus in a chain of essential growth reactions at which the inhibitor acts. An inhibitor and an essential nutrient supplied externally could clearly compete from the moment of presentation at the initial stage where the normal metabolite is taken into the biosynthetic chain. But there is no reason *a priori* why an inhibitor should not also compete with, or otherwise block, the utilization of an essential metabolite supplied endogenously by the cells' own synthesis. This could be done either (a) by blocking the utilization of the metabolite once it has been formed; or (b) by preventing its formation, *e.g.*, by a mass action effect tending to reverse the synthetic



reaction, so that the necessary rate of synthesis could not be maintained. What it is wished to emphasize here is that there is overwhelming evidence (1) of an intimate connexion between growth inhibition by sulfanilyl compounds and its abolition by *p*-aminobenzoic acid; (2) that *p*-aminobenzoic acid is synthesized by the very widest variety of micro-organisms; (3) that some micro-organisms which do not synthesize it, require *p*-aminobenzoic acid as an essential growth factor. This group of facts, seen against the background of similar findings with other essential metabolites, is too coherent for it to be argued that the prime influence of the sulfanilyl drugs is not against some physiological function concerning *p*-aminobenzoic acid.

#### 7. *Interlinking of Metabolic Reactions Concerned with p-Aminobenzoic Acid*

The effect of various compounds other than *p*-aminobenzoic acid in antagonizing the inhibitory action of sulfanilyl compounds can be accommodated within this conception, not by assuming a direct non-specific effect, but by assuming that the non-specific antagonist, *e.g.*, methionine, affects either biosynthesis of *p*-aminobenzoic acid, or the utilization of some higher stage metabolite for which *p*-aminobenzoic acid is used. Harris and Kohn (121) have examined this question. They found that methionine was, among all the natural amino acids tested, an antagonist for the inhibitory action of 4 common sulfanilyl drugs (-amide, -pyridine, -diazine, and -thiazole) on the growth of *Esch. coli*. Unlike *p*-aminobenzoic acid, methionine was effective only against low concentrations of the sulfonamides and the concentrations needed to antagonize did not show a simple relationship to the concentration of sulfonamide. It was shown that *l*(-)-methionine was 10 times more potent than *d*(+)-methionine in antagonizing sulfonamide. The analog ethionine was itself inhibitory; its action could be neutralized by methionine, but not by *p*-aminobenzoic acid. Inhibition of growth was also observed with *dl*-aminobutyric acid, *dl*-norvaline and *dl*-norleucine, the last two compounds but not the first being neutralized by methionine, and none by *p*-aminobenzoic acid. These inter-relations of inhibition and antagonism between amino acids seem to be related to similar amino acid inter-relations studied in detail by Gladstone (105). None of these amino acids, except methionine, antagonized sulfanilamide, and the effects appear to be related to a different series of metabolic reactions from those directly connected with the function of *p*-aminobenzoic acid. Only with methionine is there an evident close link with the synthesis and function of *p*-aminobenzoic acid.

Harris and Kohn (121) discussed ways in which the interlinking of reactions concerned with *p*-aminobenzoic acid utilization and methionine might cause antagonism to sulfonamide inhibitions. While their particular suggestions may or may not be correct, it is important that they have

considered the problem from the standpoint of the interlinking of the metabolic reaction chains as a whole. Their theoretical conception is therefore a flexible one and is valuable in stressing the fact that in considering (a) the biosynthetic routes whereby essential metabolites are made or into which essential nutrients are incorporated when not synthesized *in situ*; and (b) the specific inhibitions of these biosynthetic reactions, the physiology of the organism must be considered as a whole. Interference with one or a few points in the biosynthetic network necessarily affects the vital reactions as a whole and the organism may respond, directly or indirectly, by cessation of growth, or may adapt itself to circumvent the interference. One beautiful example of this interlinking has been recorded by Kohn and Harris (122). They had a strain of *Esch. coli* which grew well in an inorganic salt plus glucose medium (nitrogen source: ammonia). When sub-cultured serially in a medium containing *l*-methionine and xanthine ( $10^{-5} M$ ), glycine and *dl*-serine ( $4 \times 10^{-5} M$ ) and sulfanilamide ( $2 \times 10^{-3} M$  increasing to  $2 \times 10^{-2} M$ ), the organisms became unable to grow in the original medium. It was found that this was because the organisms had developed a need to be supplied with methionine. It would appear that the organism had lost the ability to synthesize methionine. Cultivation in presence of methionine or sulfanilamide singly did not develop methionine-requiring organisms. The authors argued that sulfanilamide inhibits some biosynthetic reactions and that of these the synthesis of methionine is among the first to be affected. To explain why cultivation in sulfanilamide alone did not change the methionine requirement, they suggested that resistance to sulfanilamide, when developed in a methionine-free medium, must involve also an adjustment to protect methionine synthesis since methionine is essential to the growth of the organism, while if methionine is present in the medium this readjustment would not be necessary. Then when this organism was transferred to the original ammonia medium it would have lost its ability to synthesize methionine. It is uncommon for an organism to lose easily its ability to synthesize an essential metabolite merely by cultivation in its presence, though some cases have been recorded. The observation that cultivation in the combined presence of sulfanilamide + methionine can produce this loss of synthetic power is very intriguing because it offers a practical approach to the study of loss of synthetic powers, which may have wide theoretical importance from the point of view of evolution. It is to be hoped that this field will be followed up.

#### 8. Resistance to Growth Inhibition Caused by *p*-Aminobenzene Sulfonamides

It has been shown that drug-resistant strains of staphylococci, obtained by cultivation in sub-bacteriostatic concentrations of sulfanilyl drugs, produce larger quantities of *p*-aminobenzoic acid than do the corresponding drug-

sensitive strains (195, 368). Microbiological methods of estimating *p*-aminobenzoic acid were used, either by the growth response of *Acetobacter suboxydans* (192) or by the specific oxidation of *p*-aminobenzoic acid using the adaptive enzymes of a soil bacillus as obtained by Mirick (246). Landy, *et al.* (195) note that not all drug-resistant strains which they have examined, *e.g.*, of *Esch. coli*, *Shigella dysenteriae*, *Vibrio cholerae*; and *Diplococcus pneumoniae*, acquired an increased ability to produce *p*-aminobenzoic acid. Thus sulfanilyl-drug resistance is not necessarily related only to increased *p*-aminobenzoic acid synthesis.

Further discussion of sulfanilyl drug inhibition would be out of place here. What the above record is designed to emphasize is the necessity for taking into consideration the whole physiology of the micro-organism when considering the findings of nutritional and growth-stimulation experiments on the one hand and of specific growth inhibitions on the other. Micro-organisms as a whole display a high degree of biochemical adaptability, so that apart from the ways in which alterations in the supply of one essential metabolite may affect the functioning of other metabolic reactions, there is the additional flexibility given by the possibility that the very mechanisms of the organism may also change. Thus the organism cannot be looked upon as a rigid mechanism into which may be fed nutrients to be turned into more of the same living mechanism constructed in the same way. The conditions under which the growth takes place may determine to a large extent the properties of the synthetic network which is built up.

#### IX. NICOTINIC ACID AND PYRIDINE NUCLEOTIDES

The isolation of the pyridine nucleotides coenzyme 1 (cozymase) and coenzyme 2 and the determination of the components of their chemical structures showed the important part which the amide of nicotinic acid played in the functioning of these substances. Before this no biological role of nicotinic acid was known, although it had been obtained (70, 379) in early attempts to isolate the then undifferentiated "vitamin B." Nicotinic acid and the related pyridine nucleotides were first implicated in a nutritional role by studies with micro-organisms. Thus the already clearly differentiated "V" factor required for growth by certain bacteria of the Hemophilus group (154, 344) was finally shown by the Lwoffs (220, 221, 222) to be either coenzyme 1 or 2, the compounds being interconvertible by the organisms. Shortly after, nicotinic acid was identified as an essential growth factor for *Staphylococcus aureus* (155, 156) and for *C. diphtheriae* (260, 261). These findings supplied a connecting link with work on the isolation of the anti-canine black tongue factor which was identified as nicotinamide (80, 81); the extension to human pellagra and subsequent developments are now well known (322). Thus observations on the metabolism

or nutrition of very different types of organism were linked up and brought about advances in apparently widely different fields. The common factor is the fact that phosphopyridine coenzymes appear to be part of the essential equipment of all cells.

*1. Micro-organisms, etc.; Requiring Nicotinic Acid  
as Growth Factor*

*a) Bacteria in General.* There are a large number of bacterial species which require nicotinic acid as an essential nutrient (Table XX). It is to be noted that not all strains of any given species will necessarily require nicotinic

TABLE XX  
*Bacteria Which Require Nicotinic Acid\* as Growth Factor*

Organism	References
<i>Staph. aureus</i>	19, 155, 156, 157, 159, 186
<i>C. diphtheriae</i>	83, 84, 260, 261, 264
<i>Proteus vulgaris</i>	88, 224a, 280, 326
<i>Proteus morganii</i>	279, 280, 281
Lactic and Propionic acid bacteria (some species and strains)	190, 250, 360, 361, 362, 366
<i>Acetobacter suboxydans</i>	392
<i>Brucellae</i>	145, 166, 167, 168
<i>Pasteurellae</i>	17, 18, 20, 165
Dysentery bacilli	64, 65, 66, 102, 168, 172. 326, 403
<i>Hemophilus pertussis</i>	132
<i>Streptococcus salivarius</i>	349
<i>Salmonella pullorum</i> (some strains)	141
<i>Esch. coli</i> , a strain	375
<i>B. mesentericus</i>	49
<i>Cl. tetani</i>	86
<i>Leuconostoc mesenteroides</i>	103a
<i>Pneumococcus</i>	10, 201

\* Nicotinic acid is taken as the key compound; for specificity see Table XXII.

acid, even if many do. Several species are known where strains which require nicotinic acid and strains which synthesize it have been found, *e.g.*, *Esch. coli*, some species of propionic and lactic acid bacteria and of *Salmonellae*. In these cases the synthesizing strains appear fairly common, while for other species, synthesizing strains are uncommon. Thus there is no well-authenticated case of *Staph. aureus* strains being able to dispense with nicotinic acid as a nutrient, although numerous strains have been examined. This is not to say that such strains may not exist naturally, or may not be deliberately produced. Strains of dysentery bacilli which ordinarily required nicotinic acid have been trained to synthesize it (172).

*b) Pasteurellae and Nicotinamide.* Strains of several species of *Pasteurella*

have been found to require nicotinamide as growth factor and to be unable to use nicotinic acid (17, 18, 20, 165). The relative effectiveness of nicotinamide and nicotinic acid have been compared for a number of bacteria (Table XXI). With the exception of *C. diphtheriae*, which uses the acid more effectively than the amide, there is a progressive increase in the effectiveness of the amide among the organisms listed. This can be interpreted as indicating a decreasing facility in synthesizing the amide from the acid. The *Pasteurella* species then represent organisms which fail to carry out this reaction to any degree compatible with growth; for them, therefore, the amide itself becomes an essential nutrient. All the organisms listed are known to synthesize "V" factor (see below).

c) *Yeasts*. In general the species of *Saccharomyces cerevisiae* which have been extensively examined for their nutrient requirements do not require an external source of nicotinic acid; they are all able to synthesize it, since they produce cozymase (423). Rogosa (320, 321) examined a large group

TABLE XXI  
*Relative Growth-promoting Effect of Nicotinamide and Nicotinic Acid (165)*

Organism	Ratio of activity amide : acid	References
<i>C. diphtheriae</i>	1 : 10	261
<i>Proteus vulgaris</i>	1 : 1	88, 165
<i>Staph. aureus</i>	5 : 1	156
Dysentery bacilli	10 : 1	66
Certain <i>Pasteurellae</i>	$\infty$ (acid ineffective)	165

The comparison of activities was made usually on the basis of 24-48 hrs. growth effect.

(114 strains) of lactose-fermenting yeasts, comparing them with 7 strains of *Saccharomyces cerevisiae* for their need for nicotinic acid. It was found that the lactose-fermenting yeasts required an external supply of nicotinic acid for growth, while the non-fermenters of lactose grew well without it.

For *Torula cremoris* Koser and Wright (171) found that nicotinamide or nicotinic acid was required (together with biotin, pantothenate, and thiamin) for prompt and abundant growth in a basal medium of inorganic salts, glucose, and ammonium phosphate. Nicotinamide and biotin supported slow growth; under certain conditions nicotinamide alone sufficed. The findings were consistent with the theory that the organism is deficient in its ability to synthesize the 4 factors mentioned, and that the addition of one or two facilitated the synthesis of the others.

d) *Roots of Higher Plants*. Nicotinic acid is essential to the growth of the isolated roots of numerous species of higher plants (1, 31, 34, 39). The specificity of the nicotinic acid structure in plant-root growth is similar to that in other organisms (34); only derivatives which yield nicotinic acid

by simple hydrolysis are active (see Table XXII). The isosteres thiazole-5-carboxylic acid and pyrazine-3-carboxylic acid were inactive.

TABLE XXII

*The Specificity of Nicotinic Acid in the Growth of Various Organisms*

Compound	References	<i>Staph. aureus</i> (158, 224a, 186)	<i>Proteus</i> (88, 211, 224a, 280)	<i>Dysentery</i> <i>bacilli</i> (66)	<i>Lacto-</i> <i>bacillus</i> <i>arab-</i> <i>inosus</i> (82)	Pea roots (34)	Dog (452)	Pellagra (82)
					Activity			
Pyridine-3-carboxylic acid (nicotinic acid)		+	+	+	+	+	+	+
Pyridine-2-carboxylic acid (picolinic acid)		—	—	—	.	—	—	—
Pyridine-4-carboxylic acid (isonicotinic acid)		—	—*	—	.	—	—	—
Methyl nicotinate		+	.	+	.	+	.	.
Ethyl nicotinate		+	+	+	+	+	+	.
Propyl nicotinate		.	.	+	.	+	.	.
Butyl nicotinate		.	.	+	.	+	.	.
Nicotinuric acid (nico- tinyl-glycine)		+	.	+	+		+	
Nicotinamide		+	+	+	+	+	+	+
Nicotinic acid methyl amide		.		.	.	.	+	.
Nicotinic acid ethyl amide		.	+	.	.	.	.	.
“ “ diethyl- amide (coramine)		—	+	+	.	.	+	+
Methyl-3-pyridine ( $\beta$ -pico- line)		—	+†	—	.	—	{ —50 mg. +200 mg.	+
Pyridine-3-nitrile		—	+†	—†	.	—		.
Pyridine-3-sulfonate		—	+†	—	.	.	—	.
Pyridine-2,3-dicarboxylic acid (quinolinic acid)		—	+	+	.	—	—	.

Derivatives of nicotinic acid which were ineffective for *Staph. aureus*, *Proteus vulgaris* and pea roots:—pyridine-3,4-dicarboxylic acid (cinchomeronic acid); pyridine-3,5-dicarboxylic acid (dinicotinic acid); 3-amino-pyridine; trigonelline; arecoline. 6-Methyl-nicotinic acid was inactive for dysentery bacilli. Derivatives differing in structure more widely were inactive.

\* Lwoff and Querido (224a) reported + effect; but Pelczar and Porter (280) found this compound negative with over 250 strains of *Proteus*.

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‡ Reported + effect with pyridine-3-nitrile using dysentery bacilli (64); this was not confirmed (66).

c) *Insects*. Nicotinic acid was found indispensable for the development of the moth *Galleria mellonella* (325). Fraenkel and Blewett (99) in an examination of the requirements of several insects for vitamins of the B-group,

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					Activity			
Pyridine-3-carboxylic acid (nicotinic acid)		+	+	+	+	+	+	+
Pyridine-2-carboxylic acid (picolinic acid)		—	—	—	.	—	—	—
Pyridine-4-carboxylic acid (isonicotinic acid)		—	—*	—	.	—	—	—
Methyl nicotinate		+	.	+	.	+	.	.
Ethyl nicotinate		+	+	+	+	+	+	.
Propyl nicotinate		.	.	+	.	+	.	.
Butyl nicotinate		.	.	+	.	+	.	.
Nicotinuric acid (nicotinyl-glycine)		+	.	+	+	.	+	.
Nicotinamide		+	+	+	+	+	+	+
Nicotinic acid methyl amide		.	.	.	.	.	+	.
Nicotinic acid ethyl amide		.	+	.	.	.	.	.
“ “ diethyl- amide (coramine)		—	+	+	.	.	+	+
Methyl-3-pyridine ( $\beta$ -pico- line)		—	+†	—	.	—	{ -50 mg. +200 mg.	+
Pyridine-3-nitrile		—	+†	—‡	.	—		.
Pyridine-3-sulfonate		—	+†	—	.	.	—	.
Pyridine-2,3-dicarboxylic acid (quinolinic acid)		—	+	+	.	—	—	.

Derivatives of nicotinic acid which were ineffective for *Staph. aureus*, *Proteus vulgaris* and pea roots:—pyridine-3,4-dicarboxylic acid (cinchomeronic acid); pyridine-3,5-dicarboxylic acid (dinicotinic acid); 3-amino-pyridine; trigonelline; arecoline. 6-Methyl-nicotinic acid was inactive for dysentery bacilli. Derivatives differing in structure more widely were inactive.

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found that *Tribolium confusum* and *Plinus lectus* both required nicotinic acid, whereas *Lasioderma serricorne*, *Silodrepa panicea*, and *Silvanus surinamensis* did not. The difference appeared due to the fact that the latter three organisms belong to the large group of insects which contain intracellular symbionts, which appear to be absent from *Tribolium* and *Plinus*, and that these symbiotic micro-organisms can synthesize growth factors, including nicotinic acid. When *Silodrepa* and *Lasioderma* were grown in the absence of symbionts (100) their nutrient requirements became more exacting. The similarity with findings concerning the synthesis of vitamins by intestinal bacteria in higher animals is evident.

## 2. Specificity

The biological specificity of nicotinic acid has been examined for several species of bacteria, isolated plant roots and in mammalian nutrition. The comparative activities of various derivatives of the parent substance are very similar for the different organisms and mainly suggest the relative abilities of these organisms to use the derivatives as sources of the parent substance for the biosynthesis of more complex compounds, of which the phosphopyridine nucleotides are known examples. There are indications that the nicotinic acid required by certain organisms is used for other purposes besides the synthesis of the known pyridine nucleotides (see p. 195). A selection of compounds illustrating the specificity is collected in Table XXII. Certain conclusions may be drawn. With the exception of pyridine-4-carboxylic acid (isonicotinic acid) which was recorded as active at  $10^{-4} M$  for *Proteus vulgaris* (224a) all the other compounds which have any activity for the bacteria examined are substituted at position 3 in the pyridine ring as in nicotinic acid itself.<sup>2</sup> The biological activity of pyridine derivatives related to nicotinic acid therefore reduces to the permissible variations in the substitution at position 3, and to the effects of additional substitutions in the ring. Full details of compounds tested and found inactive will be found in the papers cited.

There are some quantitative differences in the ability of different bacteria to use a given compound and in the ease with which different compounds can be used by the same species. Thus, among alkyl esters of nicotinic acid, the methyl ester gave delayed growth with *Staph. aureus*, the time-lag being interpreted as due to the need to hydrolyze to the free acid preparatory to amide and phosphopyridine nucleotide synthesis (159). With dysentery bacilli [3 strains of *Shigella paradysenteriae* (186)] the methyl

<sup>2</sup> Lwoff and Querido's finding (224a) is open to question since, although they used the same specimen as that which Knight and McIlwain (159) found inactive for *Staph. aureus*, Pelczar and Porter (280) found some 190 strains of *Proteus vulgaris* all unable to use the substance.

ester was readily available, but there was evidence of less ready availability with the ethyl, propyl and butyl esters, in that order (66). The diethyl amide, not utilized by *Staph. aureus* at  $10^{-4} M$ , was easily available to *Proteus vulgaris*; with dysentery bacilli the methylamide was more readily available than the diethylamide. The range of pyridine-3-derivatives utilizable by *Proteus vulgaris* appears to be somewhat wider than for *Staph. aureus* or dysentery bacilli. Thus *Proteus vulgaris* used pyridine-3-nitrile ( $10^{-4} M$ ), 3-methyl-pyridine ( $10^{-4} M$ ) and even pyridine-3-sulfonic acid (224a) which the other two species could not use. For some species of bacteria, including *Proteus* itself under the appropriate conditions, pyridine-3-sulfonic acid can act as an inhibitor of growth promoted by nicotinic acid (cf. Section VIII).

The effect of groups adjacent to the 3-carboxylic group is shown with quinolinic acid (pyridine-2,3-dicarboxylic acid) which was inactive with *Staph. aureus* ( $10^{-4} M$ ), active with *Proteus vulgaris* at  $10^{-4} M$  (224) but not at  $2 \times 10^{-5} M$  (280). With dysentery bacilli Koser, Dorfman, and Saunders (169) obtained delayed growth at  $10^{-4} M$ . Knowing the relative ease with which decarboxylation of quinolinic acid can yield nicotinic acid it is not surprising that many specimens of quinolinic acid may show some, but much decreased, activity. It is clear that the 2-carboxylic group much interferes with the utilization of the compound. 6-Methyl-nicotinic acid was inactive for Shiga dysentery bacilli.

The derivatives of nicotinic acid which are biologically active for the bacteria parallel very closely those found to cure black-tongue in dogs (82, 452). In the above, a substance has been rated as inactive when tested up to about  $10^{-4} M$  without effect, as compared with nicotinic acid or nicotinamide showing activity at concentrations of the order of  $10^{-7}$  to  $10^{-8} M$ . In some cases, however, very high concentrations of pyridine derivatives have shown effects. Thus quinolinic acid, trigonelline, and pyridine-3-carboxylic diethylamide (coramine) + nicotine, although inactive with *Staph. aureus* at  $10^{-4} M$ , nevertheless did show some activity at  $10^{-2} M$ , permitting growth after some delay (233).

### 3. Chemical Synthesis of Nicotinamide by Heat-Sterilization of Culture Media

Bovarnick (43) has found that nicotinamide may be synthesized non-biologically by heating together certain amino acids under suitable conditions, such as may occur in the heat-sterilization of culture media. Heating asparagine and glutamic acid together in presence of Mn and air yields nicotinamide, identified by isolation and characterization. These results are of importance to any conclusions which may be drawn as to the dispensability of nicotinic acid for any organism grown in heat-sterilized media.

#### 4. Growth Activity of Analogs of Nicotinic Acid

Certain compounds isosteric with nicotinic acid were examined by Koser, Dorfman, and Saunders (169) following a suggestion by Schmelkes (328). The pyrazine analogs of nicotinic acid and quinolinic acid were inactive for strains of Flexner and Sonne dysentery bacilli, but thiazole-5-carboxylic acid and its amide (isosteric with nicotinic acid) possessed low activity at  $10^{-4}$  to  $10^{-3}$  *M*, resulting in very slow growth with the same strains. It had been reported that the pyrazine compounds had caused prompt improvement in the condition of pellagrins, but later workers (57) found these compounds inactive against black-tongue in dogs, nor did they raise the "V" factor (coenzyme 1 or 2) content of human red cells when ingested.

#### 5. The Phosphopyridine Nucleotides; "V" Factor and the *Hemophilus* Group of Bacteria

The special nutrient requirements of the *Hemophilus* group of bacteria have long been recognized; two separate growth factors, the "X" and the "V" factor, were differentiated early in the study of bacterial growth requirements (58, 156, 344). The "V" factor was detected because of its heat lability under certain conditions, and the "X" factor as associated with blood pigments. The latter was shown to be an iron compound and it has been clearly demonstrated that hematin will function satisfactorily as the essential nutrient component "X" required by certain organisms of the *Hemophilus* group (210a) [not all require "X" factor]; it is physiologically effective in the respiration of these organisms. The "V" factor was not identified until 1936-1937, when it was shown by A. and M. Lwoff (220, 221, 222) that its properties corresponded with those of either of the phosphopyridine nucleotides, coenzyme 1 (cozymase) or coenzyme 2, and that either of these substances could supply the missing essential nutrient to a "V"-deficient medium and thus support the growth of *H. parainfluenzae*. Once the "X" and "V" factors had been differentiated (and before their identities had been established) the requirement for one or both of the factors was used to differentiate organisms of the *Hemophilus* group on a nutritional basis (87, 173a, 212, 294). Organisms classified as of the *Hemophilus* group on other bacteriological grounds were found separable into at least 3 subgroups: those which required "X" and "V" factors (e.g., *H. influenzae*, Pfeiffer's bacillus), "V" factor only (e.g., *H. parainfluenzae*), and "X" factor only (e.g., *H. canis*).

Fildes (87), discussing this classification of the *Hemophilus* group, suggested an organic relation between organisms of this group, the nutrient requirements reflecting changes in synthetic abilities. He regarded the sequence of development as one of gains rather than losses in synthetic power. This was one of the earliest suggestions (together with that of

Twort and Ingram (390, 391) concerning the Johne's bacillus factor, (see Section XIV)] of an evolutionary relationship between allied bacterial species which was based on changes in the synthetic abilities of the organisms, other characters of the organisms remaining the same. A systematic comparative study of physiological functions might well provide a basis for a more rational classification of bacteria. Lwoff (212) has discussed the classification problems in relation to the classification adopted in *Bergey's Manual*, and has proposed the exclusion of the two species, *Moraxella lacunata* and *M. duplex*, partly on the basis of their non-requirement of either "X" or "V" factor (9, 212). The growth factor requirements

TABLE XXIII

*Growth Factor Requirements of Hemophilus Bacteria, Showing Increasing Facility in Synthesis of "V" Factor*

Organism	References	Growth-factor requirements	
		"X" (hematin)	"V" (pyridine nucleotide)
<i>H. influenzae</i> (Pfeiffer's bacillus)	212	+	+
<i>H. conjunctivitis</i> (Koch-Weeks bacillus)	212	+	+
<i>H. parainfluenzae</i> (Rivers; Kristensen)	173a, 212, 294	0	+
<i>H. pertussis</i>	132	+	nicotinic acid
<i>H. canis</i> (Kristensen's bacillus)	173a, 212	+	0 ("V" synthesized)*
<i>H. ducreyi</i> (Ducrey's bacillus)	212	+	0 ("V" synthesized)*
<i>H. aphrophilus</i> nov. sp. (Khairat's bacillus)	148	+	0 ("V" synthesized)*
<i>H. influenzae murium</i>	212	+	0 ("V" synthesized)*

\* It is probable that the basal media used here contained nicotinic acid; it is not known whether it had a growth-promoting effect.

shown by organisms ordinarily classified in the *Hemophilus* group are shown in Table XXIII. In all cases where "V" factor is not required as nutrient, it is synthesized. This has been mainly shown by the ability of these organisms to supply "V" factor to other bacteria which require it. Throughout this review many examples are given in which increasingly complex nutrient requirements within a group indicate a decreasing facility in synthesizing an essential metabolite; the *Hemophilus* group is clearly one of these. The existence might be anticipated of organisms otherwise closely similar to characteristic members of the *Hemophilus* group, but requiring neither "X" nor "V" factor, because able to synthesize both. *H. pertussis*

is apparently such an organism; it does not require "X" or "V" factor but is classified on bacteriological grounds in the *Hemophilus* group (*Bergey's Manual*); as Hornibrook (132) has found, nicotinic acid has a growth-promoting effect. This organism thus has greater synthetic powers than those which cannot synthesize the phosphopyridine nucleotide when given the components nicotinic acid, ribose, and adenylic acid, and appears to be deficient only in its ability to synthesize nicotinic acid adequately. The classification problem cannot be further considered here; it is sufficient to stress that the nutrient requirements require to be taken much more into consideration than in the past, as an added and sensitive differentiation technique. There is much evidence that classifications which take into account nutrient requirements will be natural ones, being based on natural evolutionary relationships, and thus more rational than the classifications at present in use, which tend to be based largely on diagnostic differences observed empirically and for which no rational coordinating basis is known.

#### 6. Specificity in "V" Factor Activity

Lwoff and Lwoff (221) found that *H. parainfluenzae* was unable to use nicotinic acid, nicotinamide, adenylic acid (yeast or muscle), the diethylamide of nicotinic acid, or *o*-dihydropropyl nicotinamide. Schlenk and Gingrich (327)\* extended this, testing intermediate degradation products of cozymase. The following results were obtained:

Substance	Minimum conc. for detectable growth effect $\mu\text{-mol./ml. medium}$
Cozymase (co-enzyme 1)	$0.2 \times 10^{-4}$
Dihydrocozymase	$0.3 \times 10^{-4}$
Acid-treated dihydrocozymase	$0.4 \times 10^{-4}$
Desaminocozymase	$0.5 \times 10^{-4}$
Nicotinamide nucleoside (riboside)	$0.1 \times 10^{-4}$
Nicotinamide + <i>d</i> -ribose + adenylic acid	No growth with $1 \mu\text{-mol./ml.}$

The organism can thus synthesize the adenine portion of the molecule given the nicotinamide nucleoside moiety, i.e., given the first step of linking nicotinamide to the pentose. The somewhat lowered activity of acid-treated dihydrocozymase also indicates some degree of re-synthesis when the degradation is not carried too far. Within the above limits it appears safe to conclude that when biological material shows "V" factor activity for *H. influenzae* or *H. parainfluenzae* this is presumptive evidence of the presence of one or other of the two phosphopyridine nucleotides. The "V" factor test has been so used both in assay methods (88, 163, 164, 285) and qualitatively, to detect synthesis of "V" factor by numerous species of bacteria which do not require it as a growth factor. In all cases examined, bacteria which do not require "V" factor as an essential nutrient

\*See Gingrich and Schlenk (103b) for an extended study.

can synthesize it. The conclusion cannot be avoided that pyridine nucleotides are essential metabolites for the very widest range of bacterial species. The early observations of Grassberger (107) and others on satellite colonies of *H. influenzae* around other bacterial colonies, grown on media deficient in "V" factor, also showed a large number of organisms capable of synthesizing "V" and excreting it into the surrounding medium. This elegant technique could obviously be used rapidly to survey many species of micro-organisms for their ability to produce any of the known growth factors.

The observations of Bass, *et al.* (11), that preparations of beef and horse liver catalase could supply "V" factor activity for *H. influenzae*, were interpreted by them as real, and not due to the presence of phosphopyridine nucleotides as impurity, mainly on the results of comparative heat stability experiments. This interpretation is not yet acceptable in view of Schlenk and Gingrich's findings (327, 103b) that nicotinamide nucleoside has high "V" factor activity. A physiological function of catalase activity as replacing "V" factor activity was excluded by showing that destruction of catalase activity did not destroy the apparent "V" factor activity of the preparations. A cautious attitude to these observations is necessary until more is known about the exact composition of the catalase preparations used.

#### 7. *Physiological Function of the Phosphopyridine Nucleotides in H. parainfluenzae*

A. and M. Lwoff (222) studied the function of the two coenzymes by the use of organisms grown with limiting quantities of these substances as growth factor, thus obtaining starved organisms which were sensitive in respiration and methylene-blue reduction experiments. The interconversion of the two coenzymes within the cells under certain conditions, the using-up of the coenzymes when functioning, and specificity with regard to substrates were studied; the existence of the triphosphopyridine nucleotide in a combined state, being potentially active as a coenzyme of the oxidation system of hexose monophosphate and then unable to undergo conversion to the diphosphopyridine nucleotide, was observed. These studies of physiological function, which cannot be detailed here, show the value of a knowledge of specific nutrient requirements in preparing cells which give clear-cut responses because of their minimal content of specific nutrients [cf. Hills' experiments with thiamin-starved *Staph. aureus* (127)].

#### 8. *Differences of Synthetic Abilities of Bacteria in Relation to Phosphopyridine Nucleotides*

When some species of micro-organisms require a part, and others the whole, of the phosphopyridine nucleotide molecule, the simplest deduction

dine nucleotides, coenzymes 1 and 2; schematically this is indicated at the head of Table XXIV which shows how the increasing complexity of nutrient requirement is directly related to a decreasing facility of synthesis of the phosphopyridine nucleotides. The relative effectiveness of nicotinic acid and nicotinamide, illustrated above in Table XXI, expresses degrees of facility in accomplishing stage 2 in the above scheme. It may be that the series of organisms in Table XXI is an indication of progressive loss of synthetic ability in evolutionary development. In any case, it is clear that more complex nutrient requirements do not, in these cases, connote

the development of more complex or new metabolic reactants; the ultimate physiologically-active compound appears to be a phosphopyridine nucleotide. This does not exclude the synthesis of other unknown metabolites of which nicotinic acid forms part of the structure.

### 9. *Adaptation to Synthesize Nicotinamide*

From strains of dysentery bacilli which ordinarily require nicotinamide for growth, variants have been derived which were capable of moderate growth without added nicotinamide (172). The trained variants were obtained by serial sub-culture in presence of decreasing concentrations of nicotinamide, or by bulk inoculation of an amino acid basal medium and subsequent sub-culture on nicotinamide-free medium. The trained organisms were shown to have synthesized the V factor required by *Hemophilus parainfluenzae*, (i.e., a phosphopyridine nucleotide or something physiologically equivalent), and therefore to have synthesized nicotinic acid. In some cases the variants reverted to their original condition of requiring nicotinamide when transferred successively in glucose meat-infusion peptone broth (172). This is a good example of the relation between requirement of an essential metabolite and the organism's ability to supply it, either by synthesis or acquisition from the environment; the *nutrient* requirement reflects the ability to synthesize. The same process is exemplified on a wider biological scale by the data of Table XXI.

### 10. *Growth Inhibitors Related to Nicotinic Acid: Pyridine-3-Sulfonic Acid*

On the theory of modelling bacteriostatic substances on the structure of known essential metabolites, so that the inhibitor shall interfere with the normal utilization of the latter, pyridine-3-sulfonic acid and its amide have been examined by McIlwain (233). Here a sulfonic acid or sulfonamide group replaces the 3-carboxyl group of nicotinic acid, as the sulfonamide group replaces the carboxyl group of *p*-aminobenzoic acid (see Section VIII). The analysis of bacteriostatic effects which McIlwain observed cannot be detailed here. It is sufficient to note a few main points. Inhibitions by pyridine-3-sulfonic acid and its amide were specifically reversed by nicotinic acid or close derivatives and not by other known growth factors, including the pyridine compound pyridoxin (vitamin B<sub>6</sub>). Clearly the inhibitions were related to nicotinic acid utilization with a high degree of specificity. The organisms used were *Proteus vulgaris* and *Staph. aureus*, both requiring nicotinic acid as essential nutrient. Growth of *Proteus vulgaris* promoted by nicotinic acid was inhibited by pyridine-3-sulfonic acid, but this compound hardly affected growth promoted by nicotinamide. Indeed in the case of nicotinamide-promoted growth, pyridine-3-sulfonic acid showed the unusual effect of *accelerating*



growth. Pyridine-3-sulfonic acid alone was not capable of promoting growth, but accelerated the growth of *Esch. coli* and hemolytic streptococci under certain conditions; neither of these organisms requires nicotinic acid as growth factors, both being synthesizers. This phenomenon, which has been observed also with other inhibitory substances related to essential metabolites, is of importance for the study of the inter-relation of growth promoters and growth inhibitors, and indicates the narrow line of demarcation which exists between the growth-promoting and growth-inhibiting action of a growth factor analog. With *Staph. aureus* addition of pyridine-3-sulfonamide inhibited nicotinamide-promoted growth in a manner characteristic of competitive inhibition, but had much less effect on nicotinic acid-promoted growth.

With *Esch. coli*, which synthesizes nicotinic acid and cozymase, inhibition by pyridine-3-sulfonamide was small even at  $10^{-2}$  *M* and non-existent at smaller concentrations; this was annulled by nicotinic acid or its amide. Pyridine-3-sulfonic acid at  $10^{-2}$  *M* completely inhibited growth, but the inhibition was not annulled by nicotinic acid or amide or by cozymase. These inhibitions of *Esch. coli* were clearly of a different order from the much greater sensitivity shown by the organism which did not synthesize nicotinic acid. Here the inhibitions were clearly related to interference with nicotinic acid utilization.

From the analysis of the inhibitions McIlwain made deductions about the reactions which were inhibited. Since pyridine-3-sulfonic acid considerably inhibited the growth of *Proteus* promoted by nicotinic acid but not by nicotinamide, this suggests that the action of the inhibitor is localized at the reaction converting nicotinic acid to the amide, or at a similar stage of amide formation from a higher derivative. Growth promoted by cozymase, however, was much more strongly inhibited by pyridine-3-sulfonic acid, suggesting that nicotinamide was not used by *Proteus* solely for the synthesis of cozymase. As the formation of the necessary compounds from nicotinamide can proceed unaffected by pyridine-3-sulfonic acid, the inhibition must at least in part occur during degradation of cozymase to smaller units used in synthesis. These suggestions are mainly interesting as indications for further experimental study, by this and other techniques, of the physiological rôle of nicotinic acid in these organisms.

### 11. Physiological Function of Nicotinic Acid in Bacteria

Considerable emphasis has been placed, in previous sections, on the relation between nicotinic acid utilization and the formation of phosphopyridine nucleotide coenzymes. This has been because the latter are certainly two ultimate metabolites of which nicotinic acid forms an essential part. It is thus evident that an important part of the nicotinic acid

required as nutrient by non-synthesizing bacteria must go to build these pyridine nucleotides, in other words at least one important role of nicotinic acid in biosynthesis is known. What remains to study is the mode of this utilization. But other functions for nicotinic acid are not excluded, and indeed there are indications of rôles other than pyridine nucleotide synthesis. The time is not yet ripe for an attempt to fit all the observations together into a coherent picture, but some of the salient points will be noted, in order to show the fertility of the techniques now available, and to indicate the need for further detailed work. Dorfman, *et al.* (65) examined the relative growth response of dysentery bacilli to nicotinamide and related compounds. It was found that coenzymes 1 and 2 were less active, on the basis of their nicotinamide contents, than was nicotinamide itself, while hydrolysis of the coenzymes increased the activity to the level equivalent to the nicotinamide content. It does not necessarily follow, however, that all the nicotinamide required as growth factor was not used to build the pyridine nucleotides. It may be that preformed coenzyme, provided externally, cannot be incorporated intact into the required enzyme loci of the organisms and that there is considerable inefficiency in utilizing the preformed compound, synthesis *in situ* from nicotinic acid being a more efficient process. Using nicotinic-acid-starved dysentery bacilli, Saunders, Dorfman, and Koser (326) measured the effect of nicotinic acid and derivatives on oxygen uptake and methylene-blue reduction rate with various substrates. The more deficient in nicotinic acid, the more sensitive was the response of the cells. With washed cells of *Shigella paradysenteriae* Sonne, oxygen uptake (glucose substrate) increased with increasing concentration of added nicotinamide; nicotinamide was more effective than an equivalent quantity of nicotinic acid but as the experiment progressed the activity of the acid approached that of the amide, suggesting the conversion of acid to amide by the cells. A comparison of nicotinic acid esters showed decreasing activity in the order methyl, ethyl, butyl, propyl ester. The methyl ester was even slightly more active than the amide. On a molar basis nicotinamide was more effective than coenzyme 1 or 2; the activity of intact coenzyme 2 was lower than that of coenzyme 1. Acid hydrolysis of the latter (giving free nicotinamide rather than nicotinic acid) brought the activity up to that expected from the nicotinamide content. Similar results were obtained with *Proteus vulgaris*. The response of nicotinic-acid-starved *Proteus* cells grown with glucose in the medium was sharply different when lactate was used as respiration substrate instead of glucose. With lactate as substrate these cells did not show respiration stimulation by any of the nicotinic acid derivatives except the diphosphopyridine nucleotide; the triphospho-compound (coenzyme 2) and all simpler nicotinic acid derivatives were inactive. A quantitative determination

of whether cells which have been treated with nicotinamide or methyl nicotinate synthesize phosphopyridine nucleotide (e.g., by *H. parainfluenzae* assay) might throw light on the question of whether nicotinic acid was used by these organisms for purposes other than synthesis of the two known phosphopyridine nucleotides. In connexion with these studies of metabolism van Niel (270) stresses the importance of a paper by Morel (252, which I have unfortunately not been able to see) where it is shown that the particular period in the growth phase of *Proteus* has a profound influence on the response to nicotinamide. "During the logarithmic phase the dehydrogenase activity per cell remains constant and the metabolism of the organisms is not affected by added nicotinamide. After the inflection point the reducing power decreases by about 15% per hour, and for some time the full activity can be restored by nicotinamide". This restoration is less effective as time goes on. A metabolic "wearing-out" of coenzyme 2 could be shown in *Proteus* cells using glucose. With cells adapted to pyruvate and metabolizing only this compound, only cozymase disappeared, and its content could be maintained by adding nicotinamide. It was concluded "that the coenzymes only wear out while functioning due to an occasional irreversible change of the amide; it was calculated that such accidents took place once in every 200,000 reactions". The earlier important study of the physiological function of the phosphopyridine nucleotides in *H. parainfluenzae* by A. and M. Lwoff (222), who examined the effect of the growth substrates on the production of the two coenzymes and on their consumption during metabolism, also appears to have direct bearing on these respiration studies.

The need for consideration of the composition of the nutrient medium as a whole when examining the effect of specific nutrients on growth and metabolism is illustrated by observations of Kligler and Grossowicz (151). Using *Salmonella paratyphi* A, nicotinic acid was an essential growth factor when fermentable carbohydrate was present but not in its absence. On the basal amino acid or peptone media, the organism grew poorly, but addition of fermentable carbohydrates practically prevented growth, while sucrose, which was not fermented, had no adverse effect. Addition of nicotinic acid to the basal medium + carbohydrate permitted good growth. Clearly the nicotinic acid was important for the utilization of carbohydrate fermentation in growth, as might be expected from the need to produce phosphopyridine nucleotide coenzymes as part of the systems fermenting carbohydrates. The organisms were able apparently to use some other metabolic mechanisms, but not very effectively, when carbohydrate was absent.

Using the Thunberg tube technique, with methylene-blue, the following observations were made: with cells grown in absence or presence of added

nicotinic acid, the reduction time was the same, for lactate as substrate, whether or not cozymase was added. With addition of nicotinic acid instead of cozymase, the reduction time was significantly greater for the cells grown without nicotinic acid, *i.e.*, a longer incubation time was needed before the nicotinic acid added to the cell-suspension was converted to a form able to influence the reduction rate (e.g., coenzyme 1). Suspensions of cells grown in presence of minimal amounts of nicotinic acid activated reduction of methylene-blue more effectively than cells grown in absence of any nicotinic acid. The general conclusion was that the nicotinic acid needed to be converted to some other higher stage metabolite, *e.g.*, coenzyme 1, before it was active in carbohydrate-degradation.

Although Kligler and Grossowicz do not record many experiments with *Shigella paradysenteriae* Flexner, they state that similar results were observed with this organism. The apparent conflict with the findings of other workers that nicotinic acid is an essential nutrient for this organism may be resolved by the fact that the basal media used contained glucose, for the utilization of which nicotinic acid was needed, while the basal media without nicotinic acid were inadequate for appreciable growth by the use of other metabolic mechanisms. In any case, this example stresses once more the need to take into consideration the total composition of the nutrient medium. The components of a medium cannot be looked upon as so many discrete bricks for the building of protoplasm; they are the materials from which this is built by means of complex inter-related chains of synthesis, the utilization and efficiency of which will depend on all the materials provided by the media. Thus when different materials are provided, growth may take place by the use of different mechanisms for parts of the total biosynthesis. This variability in biosynthesis is a field which merits detailed study.

#### *12. Possible Relation of Bacteriostasis by Some Sulfanilyl Compounds to Nicotinic Acid Metabolism*

Although it appears clear that the predominant action of the anti-bacterial sulfanilyl compounds is against metabolic reactions involving *p*-aminobenzoic acid, and that in general the differences in bacteriostatic and therapeutic effects of the derivatives of the parent *p*-aminobenzene sulfonamide (*e.g.*, sulfa-pyridine, -diazine, -thiazole, -pyrazine, -guanidine) can largely be explained on the basis of their differing physical constants (15, 178a), the possibility exists that the substituent of the sulfonamide group in these derivatives may have a secondary effect in blocking utilization of some other essential metabolite. Whether this would make an important contribution to the over-all chemotherapeutic effect is another question. The possible effect of the pyridine ring of sulfapyridine against

nicotinic acid is immediately obvious, and similarly with the other substituents where the pairs: -thiazole *vs.* thiamin-thiazole, -diazine *vs.* thiamin-pyrimidine, are suggestive. There is some evidence that inhibition of certain functions in bacteria can take place because of these substituent structures. But it is necessary clearly to distinguish between such inhibition and any chemotherapeutic effect. The discussion here is confined to considering these sulfanilyl derivatives in their interference with bacterial metabolism without immediate reference to chemotherapy.

West and Coburn (414) using *Staph. aureus* found that sulfapyridine inhibition of growth was annulled by preparations of coenzymes 1 and 2 but not by nicotinic acid. Strauss, Dingle, and Finland (375), who used a strain of *Esch. coli* which required nicotinic acid for growth, and *Staph. aureus*, were unable to confirm this, but Spink, Vivino, and Mickelson (367) reported that the antagonizing effect of cozymase could be shown if the experimental conditions were correct. In all cases *p*-aminobenzoic acid was a more effective antagonist. It requires proof that the coenzyme preparations used were devoid of *p*-aminobenzoic acid contamination. With *Lactobacillus arabinosus* (requiring nicotinic acid for growth) Tepley, Axelrod, and Elvehjem (384) found that growth in a medium containing 0.5  $\mu$ g. nicotinic acid/ml. was prevented by 2.0  $\mu$ g. sulfapyridine/ml., and this was counteracted by *p*-aminobenzoic acid (0.001–0.1  $\mu$ g./ml.), or by high levels of nicotinic acid (100  $\mu$ g./ml.). Nicotinamide, cozymase, and nicotinamide-ribose nucleoside also partially reversed the sulfapyridine inhibition. Sulfaguanidine and sulfasuxidine were less potent than sulfapyridine in inhibiting *L. arabinosus*.

Direct experiments with isolated enzyme systems from yeast and liver showed no evidence of interference with the functioning of cozymase by sulfanilamide, sulfapyridine, sulfathiazole, etc. (7). Reasons were given for excluding the possibility that contaminating *p*-aminobenzoic acid in the enzyme preparation could have annulled the possible effects of the drugs. The sulfanilyl drugs have been used in metabolism experiments with washed cells of *Shigella paradysenteriae* Sonne, from cultures grown in nicotinamide-deficient media. Only sulfapyridine and sulfathiazole showed marked inhibition of the respiration-stimulation produced by nicotinamide; sulfanilamide, sulfa-guanidine, -diazine, -pyrazine, -acetamide were all practically inactive. The inhibition appeared to be competitive, and the inhibitor had to be added before the nicotinamide to produce its effect (19, 63, 68). Since *p*-aminobenzoic acid did not annul the inhibition, and since it was shown only by the pyridine and thiazole derivatives, it is clear that here was a direct interference with a nicotinic acid function. This appears to be due to some blocking effect by the pyridine and thiazole substituents; the relation of the pyridine ring to nicotinic acid is clear,

while the thiazole derivative may have isosteric similarity with nicotinic acid. It will be remembered that thiazole-5-carboxylic acid has some growth-promoting activity as a substitute for nicotinic acid (169), showing that the space structures of the thiazole and pyridine rings are sufficiently similar for them to participate in similar biological functions.

### 13. Hybrid Growth Inhibitors

The deliberate combination of two different inhibitor structures in one compound with the hope of making an exceptionally efficient drug has often been practiced in the production of chemotherapeutic substances. Sulfapyridine was not apparently made with the end in view of adding an anti-nicotinic acid group to the anti-*p*-aminobenzoic acid effect, although as just noted it may possess this quality. The relation of some of the other substituting groups in the amide group of *p*-aminobenzene sulfonamide to other essential metabolites has also just been mentioned. Positive effects against appropriate essential metabolites have yet to be shown with them. But it would appear that the hope of producing a super-efficient anti-bacterial drug by the combination of anti-metabolite structures into one molecule may be an illusory one. For in the first place it appears probable that the different essential metabolites will function at different loci within the cell, and hence it is very unlikely that one molecule could block several reactions simultaneously. At the most it might be expected to behave like an equimolar mixture of its constituent inhibitory structures *divided* by the number of these, since one molecule of the "multiple inhibitor" could probably only operate at one locus at a time. But even this might not occur, because the additional structures might make the "multiple inhibitor" molecule much less able to fit (for reasons of its physical constants and configuration) any one of the enzyme loci involved. In fact, the idea that a super-efficient molecule could be built by the incorporation of several known inhibitor structures is an ideological hangover from the time when chemotherapeutic research had to work more or less blindly and empirically, making what use it could of discoveries of "toxic" or "lethal" molecular groupings. Now that it is possible to know more specifically the targets at which inhibitors may be aimed, namely the essential metabolic reactions of the cells, it is possible consciously to design inhibitors to affect known enzyme systems.

The fallacy of the conception of a "multiple-inhibitor" molecule is well shown by the compound which McIlwain (234) made to illustrate this point, namely the hybrid molecule pyridine-3-sulfon-(2-pyridyl) amide, which is the "sulfapyridine" type analog of pyridine-3-sulfonic acid. Thus it contains the 2-pyridyl substituent in the sulfonamide group which, as shown above, has some inhibitory effect against nicotinamide-stimulated

respiration of various bacteria when given as sulfapyridine. The hybrid also contains the pyridine-3-sulfonamide grouping which acts specifically as an inhibitor of nicotinamide-promoted growth of *Proteus vulgaris* and *Staph. aureus* (233). But McIlwain found that the hybrid molecule was less active than pyridine-3-sulfonamide itself. On the general grounds mentioned above a hybrid molecule might be expected to be unusually active only if a fortunate juxtaposition of enzyme loci allowed it to block two reactions simultaneously. And even then one of these reactions might be so much more important than the other, in the metabolism of the organism, that to block an additional reaction would add nothing. To stop growth, in other words, only one key reaction may need blocking; this may give a direction to the design of chemotherapeutically effective substances. And to study the biosynthetic net-work there are also advantages in using as tools inhibitors which are modelled on one essential metabolite at a time, rather than "multiple inhibitors", the use of which may obscure rather than clarify the observations.

## X. PURINES AND PYRIMIDINES

A number of naturally-occurring purines and pyrimidines have been found of importance in the nutrition of micro-organisms within recent years. It is clear that developments in this field are only at their beginning; all that is appropriate here is to indicate some of the findings which suggest lines along which expansion may be expected.

### 1. *Uracil and the Growth of Staph. aureus*

Richardson (293) found that uracil was indispensable for the anaerobic growth of *Staph. aureus* growing in a medium of known composition; the organism also required the addition of pyruvic acid under these conditions. Aerobically neither uracil nor pyruvic acid was essential. Under aerobic conditions the organism was able to synthesize the uracil it required, as was shown by testing aerobically-grown organisms after hydrolysis; the extracts could supply material which was uracil-active in anaerobic growth. Since it was also shown that of twenty-one compounds closely related to uracil none was active, aerobic synthesis of uracil by *Staph. aureus* appears proved. It was shown in the same way that *Eberthella typhosa*, growing aerobically, synthesized uracil.

### 2. *Specificity of Uracil in Anaerobic Growth of Staph. aureus*

A detectable effect with uracil was noted down to a concentration of  $4 \times 10^{-7}$  M, while no effect was noted up to a concentration at least 100 times greater with 21 related compounds including: 4-methyl-uracil, 1,3-dimethyl-uracil, 1,3,4-trimethyl-uracil, 2-thio-5-methyl-uracil, 4-hy-

droxy-uracil, and the following purines and pyrimidines: cytosine, isocytosine, guanine, adenine. Only with a mixture of: cytosin, isocytosine, adenine, guanine (conc. in medium  $5 \times 10^{-4} M$ ) was a small effect observed, which was much enhanced by further addition of uracil itself. Thus the specificity of uracil in this case was very great (293).

### 3. Uracil as Growth Factor for Other Bacteria

Uracil has been found important in the nutrition of other bacteria.

a. *Cl. tetani*. Uracil is indispensable for growth, with a detectable effect at 0.5  $\mu\text{g.}/10 \text{ ml.}$  of medium and optimal effect at 5.0  $\mu\text{g.}/\text{ml.}$  (86).

b. *Shigella paradysenteriae* (Flexner). One unusually exacting strain (of 20 examined by Weil and Black, 403) was found by Hutner (138) to require uracil and nicotinic acid as indispensable nutrients.

c. *Streptococcus salivarius*. Although this organism grew without added uracil, the addition of uracil greatly stimulated the rate and extent of growth (349).

d. *Lactic Acid Bacteria*. Snell and Mitchell (356) found that uracil greatly stimulated the growth of *Lactobacillus arabinosus* and less markedly *Leuconostoc mesenteroides*. Interchangeability with the corresponding oxy-derivative cytosine was noted (cf. also 53a).

e. *Hemolytic Streptococci*. Uracil and adenine appear important nutrients for organisms of this group, though the degree of importance may vary. For optimal growth, these compounds (possibly with other purines and pyrimidines) are usually included in media of known composition upon which the organism will grow more or less luxuriantly (21, 277, 319a).

### 4. Other Pyrimidines and Purines as Growth Factors

Various purines and pyrimidines have been reported as important in the nutrition of several species of micro-organisms,—whether apparently as an indispensable nutrient or as a growth stimulant depends partly upon the criterion used. In any case, the distinction is largely unimportant; the degree of *nutrient* importance will be related to the total composition of the medium and to the biochemical properties of the particular organisms under the actual conditions of observation.

a) *Lactic Acid Bacteria*. Snell and Mitchell (356) found that various species and strains of lactic acid bacteria growing in media of known composition required one or more of the following substances: adenine, guanine, thymine, uracil. They observed cases where continued growth took place in the absence of added supplies of these compounds, but the rate of growth was greatly increased by their presence. Under certain conditions each of these compounds might become the factor limiting growth. Thus adenine greatly stimulated *L. arabinosus* and *L. pentosus*



and appeared essential for *Streptococcus lactis*; uracil greatly stimulated *L. arabinosus* and somewhat less markedly *Leuconostoc mesenteroides*, for which guanine was essential. Thymine was essential for *Streptococcus lactis*. A certain interchangeability was found; in general the naturally occurring amino derivatives were replaceable by the corresponding hydroxy-derivatives, e.g., uracil by cytosine, guanine by xanthine. Although in some cases guanine and adenine were interchangeable the resultant growth was less good than when the preferred compound was used.

Feeney and Strong (85) observed growth stimulation of *Lactobacillus casei* by adenine and guanine, among other compounds, under certain cultural conditions. Subsequently (357) growth-promoting effects with *Lactobacillus arabinosus* and *L. pentosus* were observed with adenine, guanine, xanthine, hypoxanthine, *p*-aminobenzoic acid and *dl*-methionine, any one of these compounds permitting growth under the conditions of testing. With *Streptococcus lactis* R and *Leuconostoc mesenteroides* the requirement for one or more of the purines was more specific; *p*-aminobenzoic acid and *dl*-methionine were ineffective. *L. mesenteroides* unlike *S. lactis* R could not use adenine; xanthine, hypoxanthine, guanine were effective. The ability of these compounds to influence the reversal of inhibitions of growth caused by sulfanilamide was observed (see below). Möller (250) found guanine or adenine increased the growth of *Streptobact. (Lactobacillus) plantarum*. In their examination of the growth requirements of a strain C 203S, of *Strept. hemolyticus* (Group A), Pappenheimer and Hottle (277) found a very interesting interdependence between adenylic acid and the CO<sub>2</sub> pressure in the gas-phase, in their effects on growth. On the medium used, no growth of the C 203S strain of streptococcus took place when no purine was present and when the CO<sub>2</sub> pressure was less than about 4 mm. Hg (atmospheric CO<sub>2</sub> pressure is about 0.4 mm.). Addition of adenine permitted growth. Adenine could be replaced by: adenosine, adenylic acid, guanine, guanosine, guanylic acid, xanthine, or hypoxanthine; but uric acid, caffeine, theophylline, and the pyrimidines uracil and cytosine were ineffective. The effect of different pressures of CO<sub>2</sub> in the gas-phase is recorded in Table XXV, which shows that a sufficiently increased CO<sub>2</sub> pressure can largely off-set a lack of adenylic acid (and presumably other of the adenine-active compounds mentioned, though this is not explicitly stated).

Whether the CO<sub>2</sub> was needed for purine synthesis or whether the purine played a part in producing CO<sub>2</sub>, which was needed for growth, was not determined. With many bacteria, CO<sub>2</sub> is essential at least for the initiation of growth (cf. 106). These observations should be compared with the relation between pyridoxin requirement and O<sub>2</sub> tension observed by Bohonos, Hutchings, and Peterson (15) (see Section IV, 3) and the uracil-O<sub>2</sub> effect found by Richardson with *Staph. aureus* (293).

b) With *Acetobacter suboxydans*, Landy and Streightoff (196) found that adenine, guanine, and xanthine increased the growth response to very low concentrations of *p*-aminobenzoic acid, which is an essential nutrient for this organism. In absence of added purines the test organism responded to 0.01  $\mu\text{g.}$  of *p*-aminobenzoic acid, but with 50  $\mu\text{g.}$  of each purine added the organism was sensitive to 0.001  $\mu\text{g.}$  *p*-aminobenzoic acid. Adenine possessed the greatest activity when tested singly; 150  $\mu\text{g.}$  could replace the 3 together, although the result was more uniform with the latter mixture. Hypoxanthine and uracil had no effect. The active purines had no effect in the absence of *p*-aminobenzoic acid, *i.e.*, the latter was indispensable under the conditions used and could not be replaced by the 3 purines. On the other hand, with more than 0.03  $\mu\text{g.}$  of *p*-aminobenzoic acid per ml. the purines showed no effect. Snell and Mitchell (357) found

TABLE XXV

*Effect of Adenylic Acid and CO<sub>2</sub> on Growth of Strept. hemolyticus C 203 S (277)*  
Pressure of O<sub>2</sub> 120 mm. Hg. Total pressure made up to 740 mm. with N<sub>2</sub>. Growth given as (mg. bacterial N/10 ml. of culture)  $\times$  50.

20 hr. and 40 hr. readings are from different sets of tubes.

CO <sub>2</sub> pressure mm. Hg	No adenylic acid		Growth with: Adenylic acid (10 $\mu\text{g.}/\text{ml.}$ )	
	20 hrs.	40 hrs.	20 hrs.	40 hrs.
0.0	—	0.3	—	2.2
0.4	0.0	0.7	1.9	6.8-8.2*
1.4	0.1	0.7	3.1	8.4
2.4	0.15	3.8	3.0	9.1
4.3	0.15	9.0	10.9	10.1
8.0	1.4	9.1	11.3	10.4
20.0	1.0	9.2	9.6	10.8
40.0	10.1	12.2	12.2	12.4

\* Variable.

an interchangeability of certain purines with *p*-aminobenzoic acid and *dl*-methionine (see below).

c) *Spirillum serpens*. Pennington (282) found hypoxanthine, or an equimolar mixture of adenine and guanine essential for the growth of *Spirillum serpens*; neither adenine nor guanine alone was effective and sufficiently large quantities of either inhibited the effect of hypoxanthine. Uric acid, xanthine, uracil, adenosine, and yeast adenylic acid were without growth stimulating effect. It was suggested that 2 purine molecules were needed to form a molecule of a higher stage essential metabolite; this complex could be formed from 2 molecules of hypoxanthine, or one each of guanine and adenine.

d) *Lower Fungi*. Robbins (296, 297, 299) found that spore germination, early mycelial growth and gametic reproduction of *Phycomyces* (at 28°) in presence of excess thiamin (essential growth factor), were markedly

benefited by various extracts from natural sources. The extracts could be separated into 2 active fractions in which were postulated 2 active factors,  $Z_1$  and  $Z_2$  respectively. Using excess of a preparation containing  $Z_2$ , the nature of  $Z_1$  was investigated. A large number of known growth factors were excluded by trial, including a number of purines and pyrimidines. Guanine however was found to be active; yeast nucleic acid was only slightly effective, but after hydrolysis the activity approximated to that of the estimated guanine content. Guanine could not be the  $Z_1$  factor itself because nitrous acid destroyed the activity of guanine but not that of  $Z_1$  concentrates; xanthine was inactive, as expected from the action of nitrous acid in deactivating guanine. Other purines and derivatives related to guanine were later tested (310), of which only hypoxanthine was active. Hypoxanthine was isolated from  $Z_1$ -active fractions from potato tubers.

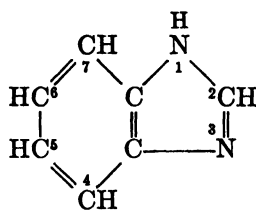
The specificity of guanine and hypoxanthine was examined (302, 310) and showed that only these two compounds, *i.e.*, guanine (2-amino-6-hydroxy-purine) and hypoxanthine (6-hydroxy-purine) were effective (equally), but isoguanine (2-hydroxy-6-amino-), xanthine (2,6-dihydroxy-), and adenine (6-amino) were inactive. Guanosine, *i.e.*, guanine with a sugar residue attached at position 7, was inactive, suggesting that the organism could not remove this residue. Its inactivity also does not support the view that the guanine was to be used for biosynthesis of nucleic acids or nucleotides. Guanylic acid was inactive, as also were 8-hydroxy-purine, 6-amino-8-hydroxy-purine and 6,8-dihydroxy-purine and 2-amino-uric acid. Slight activity was observed with 7-methyl-guanine and 1,7-dimethyl-guanine, having respectively about 1/10th and 1/100th the activity of hypoxanthine, which itself was somewhat more active than guanine.

e) *Mutants of Neurospora* have been observed which require pyrimidine nucleosides and nucleotides as growth factors. Strain 1298 appeared to require uracil (383). This and another pyrimidine-defective strain H263 were examined by Loring and Pierce (209) who found that certain pyrimidine nucleosides or nucleotides were 10 to 60 times as active as uracil; these were the nucleosides uridine and cytidine, and the corresponding nucleotides uridylic acid and cytidylic acid. Cytosine and thymine were completely inactive for strain 1298 but were slightly active for H263. Of purines and simple uracil derivatives tested only orotic acid (uracil with -COOH at position 4 instead of a hydroxyl group) had appreciable activity. The authors concluded that the difference in availability of the free pyrimidines for the growth of the 2 strains, the production of a heterocaryon when they were allowed to fuse and the types of segregation obtained after crosses with the normal wild (pyrimidine synthesizing) type were consistent with the theory that pyrimidine synthesis in these micro-

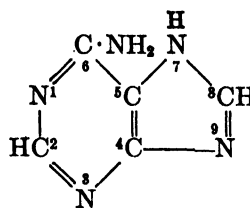
organisms involves a series of reactions each of which is controlled by a single gene.

### 5. Purines as Reversers of Growth Inhibitors

a) *Reversals by Adenine and Guanine of inhibitions by Benzimidazoles.* Woolley (446) observed growth inhibitions by benzimidazole, using yeasts and certain bacteria as test organisms.



Benzimidazole



Adenine

Because of certain structural similarities between benzimidazole and biotin, attempts were first made to reverse inhibitions by benzimidazole

TABLE XXVI

*Relative Activity of Benzimidazoles in Causing Inhibition of Growth of Saccharomyces cerevisiae (446)*

Compound	$\mu\text{g./ml.}$ required to cause half maximal inhibition
Benzimidazole	300
2-Methyl-benzimidazole	500 (not complete even with 2,000)
4-Nitro-benzimidazole	450
4-Amino-benzimidazole	400
5-Amino-benzimidazole	1,200
2-Hydroxy-benzimidazole	No effect with 500
2-Hydroxy-imidazole	" " " 500
Glucobenzimidazole	" " " 5,000

with biotin, but unsuccessfully. The structural similarity of benzimidazole to purine likewise suggested attempting to use purines for reversal of the inhibition. Of the purines tested positive results were obtained with the two aminopurines, guanine (2-amino-6-hydroxy-purine) and adenine (6-amino-purine). The specificity of the benzimidazole structure was examined (Table XXVI). With sub-inhibitory concentrations of benzimidazoles slight growth stimulation was observed with *Sacch. cerevisiae* (similar stimulation with sub-inhibitory concentrations of other growth inhibitors has been observed: cf. 233) and slightly greater stimulation by glucobenzimidazole and 2-hydroxy-benzimidazole. From the specificity studies with benzimidazoles it is seen that the H atom at position 2 must be free; substitution by a side chain or hydroxyl destroys or greatly dimin-

ishes the inhibitory activity; and the introduction of an amino group at 4 or 5, yielding derivatives somewhat more analogous to adenine and guanine, did not enhance the activity. 4-Amino-benzimidazole and 4-nitro-benzimidazole had approximately the same activity as benzimidazole on a molar basis.

A peculiarity of benzimidazole is that it is like sulfanilamide in being an inhibitor which is effective against organisms able to synthesize the natural antagonist. Other inhibitors which have been modelled on essential metabolites (*e.g.*, pyridine-3-sulfonic acid *vs.* nicotinic acid; pantooyltaurine *vs.* pantothenic acid, pyrithiamin *vs.* thiamin) are most effective against organisms which do not synthesize the natural antagonist (essential metabolite) and which therefore require an external source of supply for growth.

The relative effectiveness of benzimidazole against various organisms was as follows (446):

	$\mu\text{g. Benzimidazole/ml. to cause } \frac{1}{4} \text{ max. inhibition}$
<i>Saccharomyces cerevisiae</i>	300
<i>Endomyces vernalis</i>	300
<i>Escherichia coli</i>	370
<i>Streptococcus lactis</i> R	750

In all cases, adenine reversed the effect, except with *Streptococcus lactis* R where concentrations of adenine expected to reverse the effect were themselves inhibitory. But in this case uracil partly reversed the inhibition produced by benzimidazole.

The specificity of the purine antagonists of benzimidazole inhibition of *Sacch. cerevisiae* was as follows (446):

	$\mu\text{g./ml. required for } \frac{1}{4} \text{ max. reversal of inhibition by } 600 \mu\text{g./ml. benzimidazole}$
Adenine sulfate	600
Guanine hydrochloride	100
Muscle adenylic acid	500 gave 0.1 max. reversal
Hypoxanthine	no effect with 500
Xanthine	" " " 300
Yeast adenylic acid	" " " 500
Uracil	" " " 1000

Thiamin (10  $\mu\text{g./ml.}$ ); biotin (10  $\mu\text{g./ml.}$ ); cryst. folic acid (0.01  $\mu\text{g./ml.}$ ); *p*-aminobenzoic acid (100  $\mu\text{g./ml.}$ ) did not affect the benzimidazole inhibition.

b) *Effect of Purines on Reversals of Sulfanilamide Inhibition.* Snell and Mitchell (357) observed very interesting effects with purines on the inhibition by sulfanilamide of lactic acid bacteria. As previously mentioned, growth-promoting effects were found with *p*-aminobenzoic acid, *dl*-methio-

nine and purines, the compounds being interchangeable under the conditions of testing. With *Lactobacillus arabinosus*, marked inhibition was given by 2.0  $\mu\text{g.}$  sulfanilamide/ml., which was reversed by *p*-aminobenzoic acid at 0.3  $\mu\text{g.}/\text{ml.}$ , this being approximately 1000 times the quantity of the latter substance required to produce growth in the absence of sulfanilamide. The purines (adenine, guanine, xanthine, hypoxanthine) could not reverse this effect, even at concentrations 1000 times greater than those producing growth in absence of sulfanilamide. But when concentrations of *p*-aminobenzoic acid (0.03  $\mu\text{g.}/\text{ml.}$ ) were used which were just insufficient alone to annul sulfanilamide (2.0  $\mu\text{g.}/\text{ml.}$ ) inhibition, the further addition of the purines (1.0  $\mu\text{g.}/\text{ml.}$  or more) permitted growth. Guanine, xanthine, and hypoxanthine were also effective, but *dl*-methionine showed no effect. Similar but erratic effects were found with *L. pentosus*; the purines alone reversed the sulfanilamide inhibition, but different results were obtained when cryst. biotin was substituted for a biotin concentrate which was included in the basal medium. Using the medium containing cryst. biotin, growth-promoting effects with *p*-aminobenzoic acid and the purines were obtained at the same concentrations as when using the biotin concentrate, but the purines were then incapable, alone, of reversing sulfanilamide inhibition. With quantities of *p*-aminobenzoic acid (0.01  $\mu\text{g.}/\text{ml.}$ ) insufficient alone to cause reversal of sulfanilamide inhibition, addition of either of the purines abolished the inhibition. Thus the biotin concentrate contained a substance (probably not *p*-aminobenzoic acid for reasons given) which enabled the purines to take part in antagonizing sulfanilamide, and also sulfathiazole and sulfapyridine.

With *Lactobacillus casei*, the purines and *p*-aminobenzoic acid had no effect on growth and the organism was highly resistant to sulfanilamide bacteriostasis (1000  $\mu\text{g.}/\text{ml.}$  required for complete inhibition as compared with 2.0  $\mu\text{g.}/\text{ml.}$  required for *L. arabinosus* and *L. pentosus*). This could be taken as complementary evidence that *p*-aminobenzoic acid synthesis by this organism was efficient. The inhibition of *L. casci* by 1000  $\mu\text{g.}/\text{ml.}$  sulfanilamide was partially reversed by 0.01–0.03  $\mu\text{g.}/\text{ml.}$  *p*-aminobenzoic acid and more effectively by larger amounts; and marked effects were obtained with the purines alone at 0.1 to 1.0  $\mu\text{g.}/\text{ml.}$

Speculation on these interesting inter-relations in growth-promoting effects and their inhibitions would be premature, but these observations suggest metabolic interconnections between the roles of *p*-aminobenzoic acid and the purines. As shown in Section VIII, *p*-aminobenzoic acid is the best antagonist of the sulfanilamide inhibition; some interference with *p*-aminobenzoic acid utilization appears to be the most sensitive locus of attack by sulfanilamide. The effect of the purines may be through permitting easier *p*-aminobenzoic acid synthesis by organisms which ordi-

narily, in the medium used, find this difficult. The observations with *Acetobacter suboxydans* (196) noted above appear to indicate an inter-relation of this kind. Similarly the interchangeability of *p*-aminobenzoic acid and the purines in growth-promoting effect may be connected with synthesis. It is legitimate to assume that both compounds are essential for these organisms. The ease of biosynthesis of a particular metabolite will vary with the species (or strain) and with the ease with which other reactions supplying the means of synthesis (enzyme systems) of the particular metabolite are carried out. It might be that supplying purines makes the synthesis of *p*-aminobenzoic acid easier for the organism in this way. Other inter-relations of *p*-aminobenzoic acid and the sulfanilyl inhibitors, and less specific antagonists of the inhibitors, are mentioned in Section VIII.

### 6. Conclusions

The observations on the effects of purines and pyrimidines, as a whole, fit into the general picture relating nutrient requirements to the essential metabolic reactions required for growth, the latter being common to widely different kinds of organism. With the known importance of the nucleic acids as cell constituents, and their complexity, it is to be expected that a wide variety of partial deficiencies in biosynthesis may be encountered among micro-organisms. This will be reflected as a greater or less need for the components of nucleic acids as nutrients and growth stimulants. The greater effectiveness of certain nucleosides and nucleotides than uracil, observed with the *Neurospora* mutants, is in line with this expectation. Classification into growth essentials and growth stimulants does not appear important. What matters is the relation of the given substance to the metabolic processes of the organism, and in many cases this is connected with the organism's rate of synthesis of the given substance under the given nutrient conditions. Until the composition of the nucleic acid in micro-organisms is known, we cannot know how much the purines and pyrimidines active in growth promotion contribute to their synthesis. And there will undoubtedly be other uses to which these growth factors are put in biosynthesis (co-enzymes, etc.). Interchangeability in growth promotion will depend to some extent upon an organism's own capacity for interconverting some of these compounds and upon the degree of variability permissible in the higher-stage metabolites which they are used to construct. The need to bear in mind the interconnexions in metabolism when examining nutritional requirements is emphasized by these studies with purines and pyrimidines. It is clear that exact studies with pure compounds related to metabolites representing presumed higher stages in biosynthesis, coupled with the use of specific inhibitors, will form a

valuable technique for investigating the routes of biosynthesis which is only at the beginning of fruitful exploitation.

## XI. INOSITOL

*meso*-Inositol was one of the first specific compounds to be differentiated among the growth requirements of yeasts, when it was isolated in 1928 by Eastcott (77) as one of the components (Bios I) of the so-called "bios-complex"; since then its influence in yeast growth has been widely studied. It was next implicated in the nutrition of micro-organisms by Buston and Pramanik (50) who found that it was an essential growth factor for the fungus parasitic on cotton-bolls, *Nematospora gossypii* (*Ashbya gossypii*); other lower fungi also require it. Most recently it has been implicated in animal nutrition, being a factor in the causation of an alopecia in mice (442). At present inositol is not known as a nutritional requirement of any bacteria, although several species have been shown to synthesize it and are capable of metabolizing it. In view of these findings and, in addition, the high concentration of inositol in brain and heart muscle of higher animals and its wide natural distribution, it is clearly of wide biological importance.

### 1. Yeast Growth

Williams (423) has reviewed the question of inositol requirements in yeast growth. Since the initial discovery by Eastcott (77) inositol has appeared to have varying importance in yeast growth, but the position is now much clearer and the influence of inositol is in line with that of other essential metabolites. Yeast strains may differ in their requirements for inositol, depending on their relative abilities to synthesize it and upon the composition of the culture medium. Some strains are able to grow from minute inocula in inositol-free media; the final yeast crops then have about the normal content of inositol, having synthesized it from other compounds in the medium (423). Janssens (140) found inositol indispensable for Wildiers' yeast; "old process" yeast appears to require it for continued reproduction (425). In studies of the inter-relations of the five compounds: inositol, biotin, thiamin, pantothenic acid, pyridoxin, it was found (425) that the inositol requirement was different among the three commercial strains studied; it was increasingly important in the order: Fleischmann's bakers yeast, "Gebrüder Meyer," "Old process." Lochhead and Landerkin (207) examined similarly the nutrient requirements of a group of osmophilic yeasts of the genus *Zygosaccharomyces*. The relative importance of the different growth factors varied among the strains examined; omission of inositol was in most cases without effect on growth. Five strains representing three species required inositol for opti-



mum growth, *Zygosaccharomyces nectarophilus* being particularly sensitive. It appears in general with the yeasts that the nutrient requirements reflect the synthetic ability of the given organisms in the medium used.

### 2. Assay of Inositol by Yeast Growth and Specificity

Yeast growth has been used as an assay-method for inositol (358, 431, 443). Woolley (443) examined the specificity of inositol by the yeast growth response (Hansen No. 1 strain of Toronto yeast) and for the curative effect on mice with an alopecia due to inositol deficiency (Table XXVII). The results were of the same type as with other growth factors; apparently those compounds are active which can readily form inositol, and any marked departure from this results in inactivity, *e.g.*, quercitol, inosose, and quinic acid. Although the mytilitol was a natural product it was concluded by Woolley that its activity could not be ascribed to contaminating inositol.

TABLE XXVII

*Activity of Compounds Related to Inositol for Mice and for Yeast (443)*

Compound	Curative effect on mice Dose Activity μg./100 g. ration		Yeast growth activity %
meso-Inositol	100	+	100
Mytilitol	200	+	10
Inositol monophosphate	.	.	5
Inositol tetraphosphate	.	.	2
Phytin	100	+	1
Soybean cephalin	2,000	+	1

Inactive for both organisms: meso-inositol hexa-acetate, *l*-inositol, *d*-inositol, quercitol, quebrachitol, and pinitol. In addition Na phytate and inosose were inactive for yeast.

In contrast with yeast, the mice were able to use phytin, soybean cephalin [which contains an inositol-containing phosphatide (150)] and inositol hexa-acetate. Thus the yeast appears unable to hydrolyze these esters, and inositol cannot enter the metabolism of yeast in these forms; the mouse may hydrolyze the esters or utilize them more directly.

### 3. Bacterial Synthesis

Inositol-containing phosphatides have been reported in tubercle bacilli (8). Woolley (445) found that bacteria from the intestinal tract of mice which had exhibited spontaneous cure of inositol deficiency alopecia, synthesized inositol. This synthesis took place in presence of gramicidin, which inhibits Gram-positive bacteria; the organisms in the treated cultures were found to be Gram-negative. But *Escherchia coli*, a prominent Gram-negative organism of the intestinal tract, did not form inositol. Thompson (386) found that the following bacteria synthesized inositol: *Aerobacter aero-*

*genes* (Gram-) aerobic and anaerobic growth 1.4–1.6 mg./g. dry cells; *Serratia marcescens* (Gram-) 1.6 mg./g; *Pseudomonas fluorescens* (Gram-) 1.7 mg./g; *Proteus vulgaris* (Gram-) 1.0 mg./g; *Clostridium butylicum* (? Gram +) 0.87 mg./g; apparently no inositol was excreted into the medium. Nielsen and Black (271) observed that rats fed sulfasuxidine developed a symmetrical alopecia which was prevented in onset by feeding inositol, but not by biotin and folic acid. This may be explicable by assuming that the rats normally derived inositol from intestinal synthesis by bacteria whose growth was inhibited by sulfasuxidine.

#### 4. Bacterial Metabolism

Kluyver, Hof, and Boezaardt (153) found that *Pseudomonas beijerinckii* Hof (129) an organism causing a purple discoloration in salted beans, produced the purple pigment which was identified as the calcium salt of tetrahydroxy-quinone, from *meso*-inositol. This was isolated from the beans. The organism could also form the pigment, and more rapidly, from *l*-inositol. *Pseudomonas beijerinckii* can grow in a simple medium (at pH 8.4) containing only ammonium succinate (0.5%),  $K_2HPO_4$  (0.04%),  $MgSO_4$  (0.02%), and NaCl (12%—the organism is markedly halophilic). The pigment was produced only with the further addition of inositol. It was suggested that the organism probably oxidized inositol to triketoinositol (hexahydroxy-benzene), which was then converted by autoxidation, under conditions of restricted  $O_2$  access, to the tetrahydroxy-quinone. The oxidation of inositol to tetrahydroxy-quinone was not carried out by washed suspensions of the organism; oxygen uptake was then very slow. Some other species of *Pseudomonas* were observed to be able to metabolize inositol; *Pseudomonas fluorescens* oxidized it completely to  $CO_2$  and water. *Acetobacter suboxydans* oxidizes inositol to inosose, i.e., monoketo-inositol (152).

#### 5. Growth Factor for Fungi

Inositol was first identified as a growth factor for a fungus in the case of *Ashbya (Nematospora) gossypii* (50); Kögl and Fries (161) added thiamin and biotin to the growth requirements of this organism. There are indications that *Lophodermium pinastri*, *Trametes serialis*, and possibly *Tricholoma nudum* also require inositol for optimum growth (161). *Trichophyton discoides* requires inositol as an essential growth factor (319). This organism could utilize to some extent Ca phytate and an inositol-phosphatide from brain (95), but free *meso*-inositol was much more effective.

### XII. CHOLINE

Choline has been extensively studied as a dietary factor in mammals and much is known about its function as a lipotropic factor and its bio-

chemical behavior in relation to other components of diets, including sulfur-containing amino acids and B group vitamins; it is also important in avian nutrition [see review by Best and Lucas (23)]. It is only recently that choline has been implicated as a nutritional requirement of micro-organisms.

### 1. *Pneumococcus*

Rane and Subbarow (291) reported that choline was essential for the growth of certain strains of pneumococci (Types I, II, V, VIII) in media of known composition. Badger (10) extended these observations by examination of the specificity of the choline structure using a Type III strain. Thirty five different compounds related to choline were examined, the organism being grown on a chemically defined medium, with the exception of "vitamin-free" casein hydrolyzate as source of amino acids. The clear-cut growth responses were measured turbidimetrically. The original paper should be consulted for the extensive tables from which Table XXVIII is condensed. Analysis of the relative activities of the compounds indicates the following structural requirements. This pneumococcus requires a molecule containing a  $N \cdot C \cdot C \cdot OH$  or  $N \cdot C \cdot C \cdot C \cdot OH$  chain. Substitution of  $CH_3$  or  $C_2H_5$  on the N atom increases the activity (*e.g.*, Nos. 2, 3, 4, 5, 6 compared with No. 7) except in the cases of Nos. 15 and 17, when the activity is decreased (*e.g.*, No. 16). Addition of a phenyl or acetyl group to the N atom or a carboxyl group to either C atom inactivates the molecule. The terminal hydroxyl group must remain free; any substitution of or through this group results in loss of activity. The inactivity of: ethylamine, glycine, sarcosine, carnitine, and serine, all naturally occurring substances, suggests that choline or ethanolamine, the only naturally-occurring substances found to be active, are not required as precursors of any of the former group of compounds; nor did choline appear to be a precursor of acetylcholine, since the latter was only very slightly active, and that possibly due to slight hydrolysis. Badger considered the possible roles of choline or ethanolamine in the nutrition of this pneumococcus which were suggested by the action of choline in animal metabolism. A role in transmethylation appeared to be excluded by the activity of triethylcholine (No. 4), diethyl-ethanolamine (No. 5), coupled with the inactivity of methionine, betaine, phosphorylcholine, and other compounds containing labile methyl groups. Badger concluded that since ethanolamine can replace choline, the formation of phospholipids, which have been found in bacteria, appeared to be the most likely rôle for choline in the metabolism of this pneumococcus. There was no correlation between the activity of choline or its derivatives in promoting the growth of this pneumococcus and their action in avian and rat nutrition (252a).

**Activity of Choline Derivatives on Growth of a Strain of *Pneumococcus* (Type III) (10)**  
(Activity is recorded in % of turbidity obtained with 5  $\mu$ g. choline chloride/ml. medium = 100%)

*Substitution on N atom*

Compound	Structural formula	Activity %
1. Choline chloride	$(\text{CH}_3)_3\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$	100
2. Dimethylethanolamine	$(\text{CH}_3)_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$	100
3. Methyl-diethanolamine	$(\text{CH}_3) \cdot \text{N}(\text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH})_2$	100
4. Triethylcholine chloride	$(\text{C}_2\text{H}_5)_3\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$	100
5. Diethylethanolamine	$(\text{C}_2\text{H}_5)_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$	100
6. Dimethylethylhydroxyethyl-ammonium chloride	$(\text{CH}_3)_2(\text{C}_2\text{H}_5)\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$	100
7. Ethanolamine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$	80
8. Diethanolamine	$\text{HN}(\text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH})_2$	95
9. Triethanolamine	$\text{N}(\text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH})_3$	95
10. Tetraethanolammonium hydroxide	$\text{HO} \cdot \text{N}(\text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH})_4$	85
11. N-Acetyethanolamine	$\text{CH}_3 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$	23

*Substitution on  $\alpha$ -carbon atom*

12. $\alpha$ -Ethylethanolamine	$\text{H}_2\text{N} \cdot \text{CH}(\text{C}_2\text{H}_5) \cdot \text{CH}_2 \cdot \text{OH}$	80
13. $\alpha$ -Ethyl- $\alpha$ -hydroxymethylethanolamine	$\text{H}_2\text{N} \cdot \text{C}(\text{CH}_2\text{OH})(\text{C}_2\text{H}_5) \cdot \text{CH}_2 \cdot \text{OH}$	100
14. $\alpha$ -Methyl- $\alpha$ -hydroxymethylethanolamine	$\text{H}_2\text{N} \cdot \text{C}(\text{CH}_2\text{OH})(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{OH}$	90
15. $\alpha, \alpha$ -Dimethylethanolamine	$\text{H}_2\text{N} \cdot \text{C}(\text{CH}_3)_2 \cdot \text{CH}_2 \cdot \text{OH}$	31
16. $\alpha, \alpha$ -Dimethylcholine chloride	$(\text{CH}_3)_2\text{N}(\text{Cl}) \cdot \text{C}(\text{CH}_3)_2 \cdot \text{CH}_2 \cdot \text{OH}$	20
17. $\alpha, \alpha$ -Dihydroxymethylethanolamine	$\text{H}_2\text{N} \cdot \text{C}(\text{CH}_2\text{OH})_2 \cdot \text{CH}_2 \cdot \text{OH}$	5

*Substitution on  $\beta$ -carbon atom*

18. $\gamma$ -Diethylaminopropanol	$(\text{C}_2\text{H}_5)_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$	100
19. $\beta, \gamma$ -Propane-diol-diethylamine	$(\text{C}_2\text{H}_5)_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{OH}$	100
20. Diethylmethyl- $\beta, \gamma$ -dihydroxypropyl ammonium chloride	$(\text{C}_2\text{H}_5)_2(\text{CH}_3)\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{OH}$	20
21. Acetylcholine chloride	$(\text{CH}_3)_3\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{O} \cdot \text{CO} \cdot \text{CH}_3$	7
22. Acetyl- $\beta$ -methylcholine chloride	$(\text{CH}_3)_2\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}(\text{CH}_3) \cdot \text{O} \cdot \text{CO} \cdot \text{CH}_3$	7

*Inactive Compounds (tested up to concentration of 50  $\mu$ g./ml. medium)*

23. N-Phenylethanolamine	$\text{C}_6\text{H}_5 \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH}$
24. 2-Nitro-1-butanol	$\text{O}_2\text{N} \cdot \text{CH}(\text{C}_2\text{H}_5) \cdot \text{CH}_2 \cdot \text{OH}$
25. Serine	$\text{H}_2\text{N} \cdot \text{CH}(\text{COOH}) \cdot \text{CH}_2 \cdot \text{OH}$
26. Ethylamine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_3$
27. Ethylenediamine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH}_2$
28. Glycine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{COOH}$
29. Sarcosine	$\text{CH}_3 \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{COOH}$
30. Betaine	$(\text{CH}_3)_3\text{N} \cdot \text{CH}_2 \cdot \text{CO}$
31. $\beta$ -Methoxyethylamine	$\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{O} \cdot \text{CH}_3$
32. Carnitine	$(\text{CH}_3)_3\text{N}(\text{OH}) \cdot \text{CH}_2 \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{COOH}$
33. Phosphorylcholine chloride Ca-salt	$(\text{CH}_3)_3\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{O} \cdot \text{PO}_3\text{Ca}$
34. Urethane of $\beta$ -methylcholine chloride	$(\text{CH}_3)_2\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}(\text{CH}_3) \cdot \text{O} \cdot \text{CO} \cdot \text{C}_6\text{H}_5$
35. Carbamyl choline chloride	$(\text{CH}_3)_3\text{N}(\text{Cl}) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{O} \cdot \text{CO} \cdot \text{NH}_2$

## 2. Mutant of *Neurospora crassa* 'Cholineless'

A mutant strain which arose from a culture of wild type *Neurospora crassa* after ultra-violet irradiation, was found (133) to be unable to grow on a medium containing only salts, sucrose, and biotin which supported the growth of the wild type organism. Addition of a mixture of water-soluble growth factors permitted growth, and choline alone permitted growth when the components of the mixture were tested separately. In an examination of the specificity of choline for this mutant the following results were obtained: only choline (approx. maximal effect at 1  $\mu\text{g.}/\text{ml.}$  medium) and methionine (only 1/500 of the activity of choline) and lecithin showed activity. With pure lecithin 50% of the available choline was utilized in 3 days. The following substances were inactive: ethanolamine, dimethylamine, trimethylamine, tetramethylammonium chloride, sarcosine, betaine, creatine, and 11 water-soluble vitamins and 22 amino acids. The activity of methionine suggests that this fungus may, in part, show some of the physiological mechanisms which relate choline and methionine in higher animals.

## XIII. OLEIC ACID

Definite evidence has recently been found that oleic acid plays a part in the growth requirements of several micro-organisms. Fleming in 1909 (94) observed that the acne bacillus (*Corynebacterium acnes*), which is difficult to cultivate, grew best in ordinary nutrient agar to which 1-5% oleic acid was added.

a) *C. diphtheriae*. Cohen, Snyder, and Mueller (55) found that certain strains of *C. diphtheriae* would not grow on a medium of known composition, including all the known growth factors required by the organism, when using small inocula from freshly isolated cultures. Addition of serum caused prompt and heavy growth; milk and commercial casein were also sources of the required additional material. Fractionation of casein showed that 2 factors at least were involved; one was shown to be oleic acid. There was a relatively sharp optimum at approx. 1 mg. oleic acid/20 ml. of medium, using colony growth on agar plates as test object. Linoleic acid was inactive, as also was dihydroxystearic acid prepared from active oleic acid. Regeneration of oleic acid from inactive dihydroxystearic acid (*via* bromination and reduction) yielded oleic acid which was active. Thus, short of testing a sample of synthetic oleic acid it is proved that oleic acid and not a concomitant impurity is the active compound. Not all strains of *C. diphtheriae* responded to the 2 factors (*e.g.*, a Park 8 strain); the sensitive strain was of *gravis* type.

b) *Erysipelothrix rhusiopathiae*. This also appeared to require oleic acid as an essential growth factor; 0.2 mg./10 ml. medium was inhibitory,

but in the presence of saponin (0.3%) 0.3 to 0.6 mg. oleic acid/10 ml. medium gave good growth when the basal medium was otherwise satisfactory (132).

c) The fungus *Pityrosporum ovale* requires oleic acid as an essential growth factor when growing on a medium of known composition ( $\text{NH}_4\text{NO}_3$ , salts, glucose); 1 mg. oleic acid/10 ml. medium gave a slight effect with increasing growth up to 100 mg. oleic acid/10 ml., but even then the growth was poor. The further addition of asparagine (0.5%) permitted good growth in 1–2 weeks, heavy in 3–4 weeks. Pyridoxin and thiamin showed stimulation of growth but were not essential (16).

d) *Cl. tetani* requires oleic acid at an optimal concentration of 0.01 mg./10 ml. of a medium of known composition (86).

It is clear that oleic acid enters into the growth requirements of a sufficiently wide variety of micro-organisms to make it not unlikely that it will be found of general importance. The variation of concentration required, from about 100 mg./10 ml. medium with the fungus *Pityrosporum ovale* to 0.01 mg./ml. for *Cl. tetani* suggests that it may be used for quite different functions in these organisms. This does not exclude also some common functions.

#### XIV. NAPHTHOQUINONES

##### 1. Growth Effects with John's Bacillus

*Mycobacterium paratuberculosis* (John's bacillus) was one of the first bacteria for which a special growth factor was clearly differentiated. Twort and Ingram (390, 391) showed that other acid-fast bacteria, including *Mycobact. tuberculosis hominis* and *Mycobact. phlei* in growing produced the growth factor(s) which John's bacillus required; crude concentrates of growth factor, indicating a certain solubility in fat solvents, were prepared from *Mycobact. phlei*, but no specific compounds were identified. Not until 1940 was any further work recorded on the nature of this growth factor; then Woolley and McCarter (451) made striking observations on the effect of phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) and of 2-methyl-1,4 naphthoquinone. Synthetic phthiocol (0.1  $\mu\text{g.}/20$  ml. medium) and 2-methyl-1,4 naphthoquinone (0.1  $\mu\text{g.}/20$  ml. medium) caused marked increase in mass of growth compared with that on the control synthetic medium.

Partially purified concentrates from cells of *Mycobact. phlei* (acetone extraction $\rightarrow$ ether $\rightarrow$ water) added at a level equivalent to 1% *M. phlei* cells, and thus supplying 200  $\mu\text{g.}$  of solids/20 ml. medium, gave excellent growth. It was clear that, although the two naphthoquinones made an important contribution to the nutrition of the organism, the *M. phlei* extracts contained additional growth stimulants.

The basal medium used by Woolley and McCarter contained only glycerol, asparagine, citrate, and minerals (126). The *Mycobacteria* as a group are in general capable of growing on simple media of this type, after a more or less prolonged process of adaptation, but it is clear that these bacteria when growing in host organisms probably use nutrients of a more complex character. The process of inducing them to grow *in vitro* on simple media appears to consist in training them to synthesize for themselves substances which they would otherwise obtain from the tissues of their hosts. John's bacillus is an outstanding example of this, since it is very difficult to train to grow on simple media without the Twort-Ingram factor(s), but it has been done (71). These organisms, even on rich media, are slow growing and not easily killed under adverse conditions and so are favorably organized for changing their nutrient requirements through alterations of synthetic ability [for discussion of nutrition of *Mycobacteria* see (154)]. As mentioned in the introduction, Twort and Ingram (391) were the first to suggest that increase in the complexity of nutrient requirements might be due to losses of synthetic power, and that this might be related to the acquisition of a parasitic habit during evolutionary development. It was for these reasons that they examined *Mycobact. phlei*, a common organism found on Timothy Grass, as a source of the growth factor which they assumed *Mycobact. paratuberculosis* had lost the power to synthesize owing to its parasitic habit in the intestinal tract of cattle.

Phthiocol was isolated from tubercle bacilli (269a) and since the John's factor was soluble in fat solvents and water, Woolley and McCarter tested phthiocol for activity. The vitamin K activity of phthiocol suggested a test of other vitamin-K-active substances, and a highly potent concentrate of vitamin K, and also the simple 2-methyl-1,4-naphthoquinone were thus found to be active for John's bacillus.

## 2. Bacterial Syntheses of Naphthoquinones and Vitamin-K-Active Substances

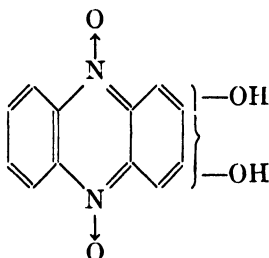
Bacterial synthesis of vitamin-K-active substances has been reported; thus *B. mycoides*, *B. cereus*, *B. subtilis*, *Sarcina lutea*, *Serratia marcescens* (*Chromobact. prodigiosum*), *Staph. aureus*, *Esch. coli*, and *Mycobact. tuberculosis hominis*, grown on complex bacteriological media, synthesize vitamin-K-active substances (5); and *Esch. coli* grown on a synthetic medium containing only glucose, citrate, asparagine and minerals produced active substances equivalent to 1000 K units/g. dried cells and excreted into the medium 400–700 K units/liter (56). It would be interesting to know whether John's bacillus, adapted to grow without added growth factors, does in fact synthesize vitamin-K-active substances.

Thus vitamin-K-active substances, of which phthiocol is a natural

product of a *Mycobacterium*, are on the one hand products of bacterial synthesis, and on the other, important in the nutrition of Johne's bacillus. It may be expected that some naphthoquinone may be found to have importance in the metabolism of many bacteria. Although no physiological function is yet known for such a compound in these organisms, support for this conclusion is given by the observation that certain naturally-occurring growth inhibitors, themselves products of bacterial growth, may be reversed by suitably constituted naphtho- and anthraquinones. On the basis of the theory that growth inhibitors act by interference with essential metabolic reactions, reversal of inhibition may be an indication of the existence of a product important in cell metabolism, (cf. the discovery of the rôle of *p*-aminobenzoic acid, Section VIII).

### 3. Naphthoquinones and the Antagonism of Iodinin Inhibition of Bacterial Growth

*Chromobacterium iodinum* (59) produces a purple pigment "iodinin" (54) which can inhibit the growth of certain bacteria, of which *Strept. hemolyticus* is the most sensitive;  $0.8-1.2 \times 10^{-6}$  *M* iodinin can prevent growth for 6 days (239). Iodinin is the di-*N*-oxide of a dihydroxyphenazine:



the positions of the OH groups are unknown, but the 2,3 and 2,5 compounds are excluded (54).

McIlwain (239) sought naturally-occurring compounds capable of reversing the inhibition of the growth of *Strept. hemolyticus* by iodinin. Such compounds were found in extracts of *rheum*; this led to the testing of anthraquinones of which the 1,4-, 1,5-, and 1,8-dihydroxy-anthraquinones were found to be active in reversing inhibitions by iodinin (at  $2 \times 10^{-6}$  *M*) at concentrations of the order of  $5 \times 10^{-7}$  to  $5 \times 10^{-6}$  *M*, depending on the particular compound. Since anti-iodinin compounds were present in normal serum, in which anthraquinones were not known to occur, partial separation of the serum compound was carried out; it was found to be soluble in fat solvents. The one group of fat-soluble substances which had been implicated in bacterial nutrition at that time was



the group of vitamin-K-substances found by Woolley and McCarter for Johne's bacillus, which McIlwain therefore tested. Vitamin-K-concentrates and the water-soluble 2-methyl-1,4-naphthoquinone were found to be active in annulling the inhibition caused by iodinin; 2-methyl-1,4-naphthoquinone was even slightly more active than the active hydroxyanthraquinones previously examined.

Discussing the mode of action of iodinin and its antagonists, McIlwain (239) concluded that iodinin was inactivated by reduction, by the organism alone when a sub-inhibitory concentration of iodinin was present, or by the organisms + the antagonist anthraquinone or naphthoquinone, when larger concentrations of inhibitor were used. The peculiarity of the antagonistic quinones lay in (a) their greater potency than other redox systems, (b) the probability that compounds with vitamin-K-activity are responsible for the antagonistic action of serum and (c) the fact that the quinones are not known to function in a manner comparable with that of, *e.g.*, methylene blue or pyocyanin in other redox systems.

Since such low concentrations of iodinin are anti-streptococcal it appears probable that iodinin interferes with specific processes in the inhibited organisms. There is a structural relation between iodinin and its most potent antagonists, and their interaction with it and with the organisms may be similar to that for other structurally related groups of inhibitors and their corresponding antagonists. In several cases the natural antagonist is intimately concerned in the metabolism of the organism against which the inhibition acts; *cf.* *p*-aminobenzoic acid and sulfanilyl drugs; amino-sulfonic acids and amino-carboxylic acids (234a, 237); related amino acids (105), *etc.* In the case of iodinin the shape and polarity of the antagonist molecule is very like that of the inhibitor, with different atoms substituted in important groups. McIlwain (239) suggested, by analogy, that iodinin formed some type of combination involving *N*-oxide groups with sites in an enzyme essential to the growth of the bacteria, these sites being involved in a reaction of the enzyme with compounds similar to those quinones found to be antagonists of iodinin. Since vitamin-K-active substances are synthesized by many bacteria, including phthiocol by *Mycobact. tuberculosis hominis*, since Johne's bacillus is stimulated in growth both by phthiocol and 2-methyl-1,4-naphthoquinone, and in view of the antagonism of iodinin-inhibition by these substances, it is clear that a search in a wide variety of microorganisms for the physiological function of some system using substances related to 2-methyl-1,4-naphthoquinone (as the simplest of the relevant known compounds) is worth making. Johne's bacillus may be an organism which is unable to synthesize the quinone required in this postulated metabolic system. Hence Johne's bacillus might be very susceptible to inhibition by iodinin or related inhibitors.

McIlwain (240) has used the structural relationship between iodinin and the antagonistic quinones as a model for preparing quinoxaline di-*N*-oxides related to compounds of anti-hemorrhagic activity. The di-*N*-oxides were inhibitory to bacterial growth, the parent diazines were inactive. These inhibitors were less active than iodinin.

### XV. EPILOGUE

The material collected in this article is representative of the large amount of knowledge about essential metabolites which has been secured, mainly during the last ten years, by the biochemical study of micro-organisms. It is clear that the initial stage of discovering essential metabolites by recognizing and characterizing growth factors is now passing to a higher stage. More growth factors will be found, but in a sense the discovery of new growth factors is becoming a routine search, in objective, if not in methods of isolation and identification.

The emphasis can now be placed on the purposeful study of the physiological functions of essential metabolites. Here the techniques which can be used are much enriched by the possibilities now available for modelling chemical structures related to essential metabolites. The power of synthetic organic chemistry can be exploited in a rational way to prepare compounds for the study of physiological function and biological flexibility,—the real biochemistry of growth. Analogs, variants and partial structures of essential metabolites can be used to study stages of biosynthesis directly. Correspondingly, specific inhibitors modelled on the structures of these metabolites increase the range of compounds available for the analysis of the biosynthetic network. Such studies with micro-organisms are of the widest value since the findings, reflecting the underlying unity of all biochemistry, will have application in all biological fields.

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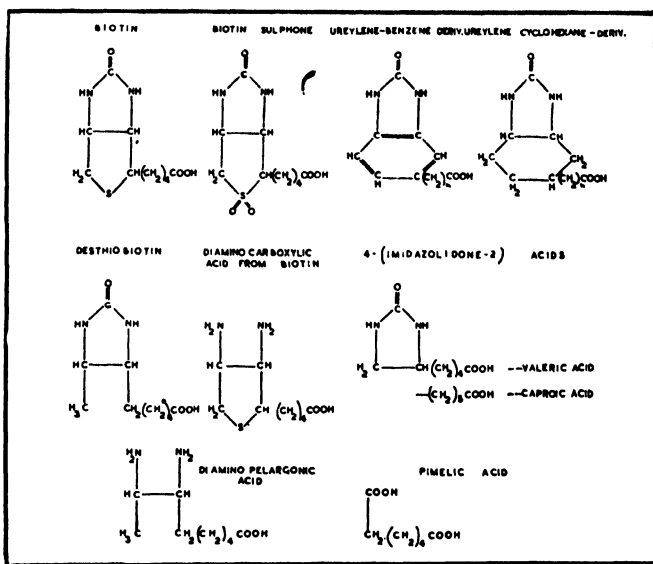
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*Footnote added in proof, to p. 116.*

André Lwoff (210) in 1932 clearly expressed the conception (particularly in connection with protozoa) that more complex nutrient requirements were a reflection of more restricted synthetic abilities, as an evolutionary loss of physiological function; Knight (154) independently reached similar conclusions for bacteria. Twort and Ingram (390, 391) had foreshadowed this in their brilliant conception already quoted. Recently two excellent surveys by the Lwoffs have become available from liberated France, unfortunately too late for anything more than citation (see 210b, 226a).

Note for Chapter VII, 6 p. 168, added in proof.

Dittmer and du Vigneaud (62a) compared the yeast growth-promoting activities of: biotin (100%); desthiobiotin (100%); the diaminocarboxylic acid obtained by opening the urea ring of biotin by barium hydroxide hydrolysis (10%); diaminopelargonic acid (10%); the urea ring opened as in the previous compound and the sulfur atom also removed as in desthiobiotin), and observed the corresponding relative activities shown in brackets. With *L. casei*, only the diaminocarboxylic acid derived from biotin showed any activity (less than 0.01%) as compared with biotin itself. The suggestion is that the yeast could use the less active compounds as less efficient



building-stones for the synthesis of the ultimate essential metabolite (biotin or a higher stage), the synthetic ability of *L. casei* being more restricted (62b).

A study was made of the action of the antibiotin factor, avidin (62a), which indicated that the urea ring portion of biotin must be intact for combination between biotin and the antibiotin protein to take place; the aliphatic side chain also seemed necessary.

Growth-inhibitions by compounds structurally related to biotin have been observed. These are different in kind from the action of the antibiotin factor, where an inactive protein complex is formed. These structurally related anti-metabolites include biotin sulfone and desthiobiotin, their activities being related to the nutritional requirements of the organ-

isms against which they act. Biotin sulfone inhibited *L. casei*, *L. arabinosus* and *Staph. aureus*, the inhibition being specifically reversed by biotin. But with *Saccharomyces cerevisiae*, which, as indicated above, has less need for biotin as a nutrient because of greater synthetic powers, biotin sulfone could even act as a growth-factor, replacing biotin, although with less efficiency (62c). Desthiobiotin also showed inhibitory effects against the growth of *L. casei* promoted by biotin; growth due to  $0.82 \times 10^{-10}$  M biotin was decreased to one-half by  $2.3 \times 10^{-8}$  M desthiobiotin, the inhibition being annulled by more biotin to a final concentration of  $4.1 \times 10^{-10}$  M (62b). Two analogs of desthiobiotin:—4-(imidazolidone-2-) caproic acid and the corresponding -valeric acid derivative were also studied as anti-metabolites (62a). These compounds differ from desthiobiotin in lacking the  $\text{CH}_3$  attached to the imidazolidone ring, and the caproic acid derivative has one more  $-\text{CH}_2$  in the side chain than in biotin. The caproic acid derivative had inhibitory effects against *L. casei* and *S. cerevisiae*, annulled by biotin. In contrast, the valeric acid derivative stimulated the yeast, but not *L. casei*, and thus extends the range of compounds which the former organism can use, probably to synthesize biotin.

Very recently two series of ureylenebenzene and ureylenecyclohexane derivatives related to the biotin structure (see formulae) have been found to possess specifically anti-biotin effects (82a) and thus provide further examples of deliberately modelled anti-metabolites.

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# Possibilities in the Realm of Synthetic Estrogens

By E. C. DODDS

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### I. THE VALIDITY OF LOGIC IN CHEMOTHERAPEUTIC RESEARCH

Of all branches of research, chemotherapy has the reputation in the eyes of laymen of being one of the most scientific and logical of subjects. It is the object of this review to question the validity of this assumption. It is also proposed to raise a number of questions, the correct answers to which would indicate whether various lines of research were worthy of prosecution.

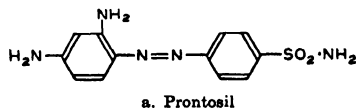
The history of all chemotherapeutic researches can be divided into two parts. Firstly, the finding or stumbling upon a clue to the structure of a substance likely to be of value, and secondly, the development of variants with a view to finding the most potent substance. The most important part of the whole research is of course the discovery of the clue; this may be purely empirical or it may be the result of making compounds of a similar or allied structure to that of the naturally occurring substance.

Many examples can be given but most of these are old history. For instance, the first classical example is the development of Salvarsan by Ehrlich. Starting with the old knowledge that arsenic was of value in cases of syphilis, attempts were made to combine arsenic with a dye on the assumption that a substance capable of staining a microorganism would be fixed by it and if, so to speak, a poisonous charge could be attached to the dye, this would be dragged into the interior of the parasite which would subsequently be destroyed. Examples could be given in the case of the phenolic antiseptic substances. As can be seen from such a study, simple logic works out very nicely up to a certain point, and an orderly series of compounds is obtained. However, as soon as a theory connecting structure with biological action is evolved, disastrous exceptions at once arise to upset the whole theory.

Perhaps the most scientifically amusing example of this apparent orderliness can be seen in the researches leading up to the development of the sulfanilamide series of drugs. Working on the old Ehrlich doctrine—*non agunt nisi fixata*—German investigators at I. G. Farbenindustrie evolved



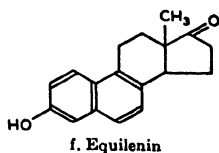
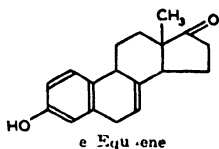
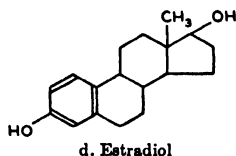
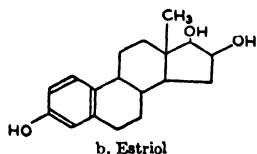
a red dyestuff, Prontosil, of the following formula (a). Here we have a very complicated molecule securely covered by patents which really gave a monopoly for the treatment of septicemic conditions. French workers, however, showed that when administered to rabbits, this substance was partly excreted in the urine as sulfanilamide, and they subsequently showed



that this was as effective as the original Prontosil. Here we see years of work on the part of the German chemists in building up this complicated molecule all being undone by the metabolic processes of the rabbit which extricates the relatively simple substance sulfanilamide from its elaborate trappings. The development of the sulfanilamide series of drugs became merely a question of working every type of substitute and finding by animal and clinical experimentation which was the better. Later the work of Fildes and his colleagues (see the review of Knight in this volume) provided a truly logical method of investigating the activity of drugs of this type.

Another series of compounds illustrating the relationship between structure and function is found in the pressor substances related to adrenalin. It is here for the first time that the chemotherapeutic principle was applied to substances actually produced by the body and utilized for influencing the functions of the body.

Perhaps the most interesting questions, however, in this problem arise in relationship to the sex hormones. This is not the place to give a complete summary of the development of our knowledge of this subject. By the early 1930s the constitution of the naturally occurring estrogens had been deduced and they are five in number; estriol, estrone, estradiol, equilene, and equilenin. The formulae are reproduced below (b, c, d, e, f).



For the first time it appeared that the body produced a series of substances capable of producing the same biological reaction. In the case of the other hormones, only one substance had been isolated. Thus only adrenalin had been prepared from the suprarenal glands, only thyroxine had been obtained from the thyroid, and only one form of crystalline insulin from the pancreas. Admittedly, synthetic products had been obtained capable of reproducing most of the reactions of adrenalin, and di-iodothyronine was found to have qualitatively the same biological properties as thyroxine although quantitatively very much less. Also in the case of the body estrogens there were five substances all possessing the same qualitative reaction but again differing in their quantitative activity, estradiol being the most active, weight for weight.

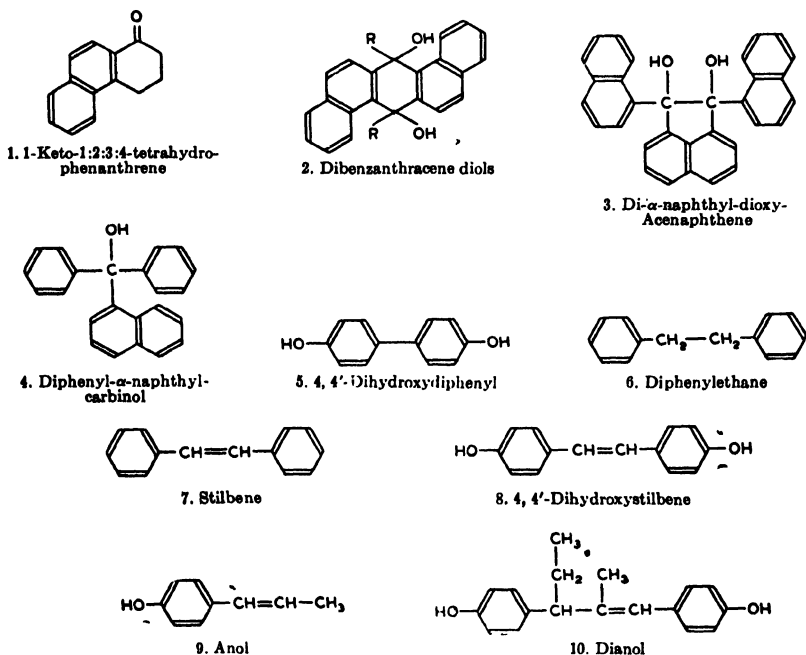
## II. FROM ESTRADIOL TO STILBESTROL

The problem as to how far one could depart from the structure of estradiol arose to the present writer and his colleagues. First an attempt was made to imitate the action of the estrogenic agents with synthetic organic compounds, derivatives of phenanthrene. The first compound to have qualitatively the same activity as the naturally occurring estrogens was found to be the substance 1-keto-1:2:3:4-tetrahydrophenanthrene (formula 1 in the scheme below). Again it is not proposed to review in detail the laborious series of investigations that followed the discovery of the activity of this compound (see (1) to (6)). For the purpose of this review we can indicate the salient points. A very large number of derivatives of phenanthrene were studied from the biological standpoint and found to possess degrees of activity varying from one-millionth to one-fiftieth of that of estriol. The next step was to decide whether the phenanthrene ring system was necessary. It was shown that certain derivatives of acenaphthene possessed estrogenic activity (7, 8). Proceeding to further simplification triphenyl carbinol was shown to be inactive whereas diphenyl- $\alpha$ -naphthyl carbinol (formula 4) was found to possess activity. Again proceeding with this simplification, the two-ring compound 4:4'-dihydroxydiphenyl (formula 5) was found to possess activity. In view of the activity of the carbinol mentioned above, the activity of substituted methane and ethane compounds was studied. It was shown that diphenyl-ethane (formula 6) possessed activity which was increased if 2 hydroxyl groups were introduced into the 4:4'-position. If the compound were desaturated and a double linking put between the two central carbon atoms the hydrocarbon diphenyl-ethylene or stilbene results, which was also found to possess activity (formula 7), and in a greater degree than the corresponding ethane derivative. The 4:4'-dihydroxystilbene (formula 8) was found to have still greater activity than the parent hydrocarbon (11).

The problem now arose as to whether one could leave out one of the

benzene rings and substitute it by a methyl group. The substance parahydroxypropenylbenzene or anol (formula 9) would obviously be the substance whose activity would be of interest. This was prepared very easily by the demethylation of anethole and was accomplished by heating this methoxy compound in a sealed tube with alcohol and potassium hydroxide. The resulting phenol was separated by the standard method and its activity was investigated. The first specimen prepared was found to have a very high degree of activity—in fact approaching that of estrone itself. This fact was published (9, 10). Shortly after publication, however, it

SCHEME SHOWING SERIES OF COMPOUNDS IN ORDER LEADING FINALLY  
TO STILBESTROL



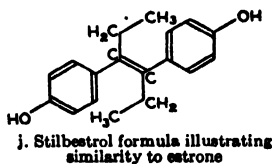
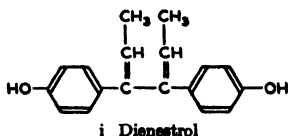
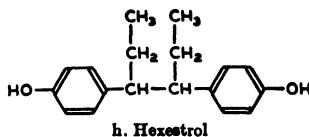
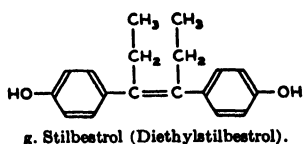
was found that the activity of all samples of anol was not identical, some being extremely active and others practically without activity, and it became obvious that something was seriously wrong in the whole conception. An investigation of the activity of the mother liquor from which the anol had been crystallized showed it to contain a glassy substance of roughly constant activity of about  $\frac{1}{100}$  that of estrone. This material was found to be very difficult—in fact impossible—to crystallize. A study of the properties of anol indicated that it polymerized very rapidly and a possible assumption was that the activity in the highly active specimen

of anol must be due to a contaminant—almost certainly a polymeride of anol. It was natural to think of the dimerides of anol and attempts were immediately put in hand to synthesize all the possible dimerides. Dianol (formula 10) had already been described; this was prepared and tested, but found to have an activity of an order that could not explain the nature of the contaminant. A symmetrical dimeride was obviously the most likely choice. Sir Robert Robinson and his co-workers at Oxford had been interested in the synthesis of chrysene and bodies more closely resembling the polycyclic estrogens, and it was decided that the workers in the writer's department and those at Oxford should combine. As a result of this collaboration a symmetrical dimeride was synthesized in 1938 and was proved to be very highly active. This substance, whose formula is shown below (g), is known as diethylstilbestrol, the term stilbestrol being used for the parent substance 4:4'-dihydroxystilbene. In Great Britain the substance is commonly known as stilbestrol, although it should, of course, be referred to as diethylstilbestrol.

As a result of careful study of the mother liquors from the anol crystallization after demethylating anethole it was possible to isolate an active contaminant, namely hexestrol, which proved to be hydrogenated stilbestrol. This possessed the formula (h) which is shown below. Another similar compound was obtained by synthesis, namely dienestrol, whose formula is shown at (i). We have, therefore, the three synthetic estrogens—stilbestrol, hexestrol, and dienestrol (11, 12, 13).

### III. THE PROBLEM OF ACTIVITY IN THE ESTROGENS

Whilst it is possible to write the formulae of stilbestrol and other synthetic estrogens in such a way that they bear a rough resemblance to the naturally occurring estrogens as shown by the diagram (j) from the strictly chemical point of view, literally the resemblance is nonexistent. Instead we are faced with the interesting problem of an extremely complicated biological reaction such as the action of naturally occurring estrogens,



embracing as it does important biological reactions on the whole of the female genital tract, the secondary sexual characteristics such as development of the breasts, external genitalia, etc., the conferment of female sexual instincts, the action on the pituitary and many other biological reactions, being carried out by two groups of substances—first by those produced in the body of a complex steroid type, and second by the relatively simple derivatives of hexane. It would appear impossible to correlate the biological activity with the chemical structure. In this laboratory and in many others attempts have been made to modify the structure of the three synthetic estrogens—namely stilbestrol, hestrol and dieneestrol with a view to increasing their activity, but any alterations in their structure such as the introduction of other hydroxyl groups into the benzene rings, modification of the length of the side chains, etc., only result in a reduction in activity.

It is very difficult indeed to interpret the significance of these observations but it would appear to the writer that the most important question is to know whether the three synthetic estrogens mentioned above represent the most active substances that can be obtained. If we return to our first introductory remarks—namely the purely logical and scientific approach to these problems, we are at once confronted with the undeniable fact that these three substances, whilst apparently resulting from a planned and orderly research, really resulted from an error of judgment and that the path which led logically to anol—which incidentally proved to be of very low activity—was by chance led from anol to stilbestrol.

Is it possible that there are other synthetic estrogens of an entirely different character and constitution still waiting to be discovered? If this applies to the estrogens, it almost certainly applies to many other biologically active substances, and it may be that bold and empirical experimentation on these lines will lead to the synthesis of an even simpler type which will really give an answer to the question of structure in relation to function.

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# Chemistry of Anti-Pernicious Anemia Substances of Liver

By Y. SUBBAROW, A. BAIRD HASTINGS, AND MILTON ELKIN

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## I. FOREWORD

The present review has for its purpose the presentation of the progress made since 1926 toward the isolation and identification of the anti-pernicious anemia material of liver. Unfortunately, within a short time, save for its historical interest, this review may be without scientific value. This arises from the fact that any hypothesis put forward by any author cannot be confirmed or denied except by experimentation on human subjects with pernicious anemia. In the course of a year the number of human subjects available for such experiments are not many. So it is only possible to evaluate the various preparations when active material is identified and is available in sufficient amounts for other investigators to confirm the data published.

As there are, at the present time, several divergent points of view regarding the chemical nature of the active material, it has seemed expedient to adhere insofar as possible to a historical presentation of the subject. Even this plan of presentation has not been rigidly followed, however, because in the interest of clarity, it seemed wise to describe the work of each group of investigators in its entirety before passing to that of another. It is hoped that in this way the reader will be able to follow better the reasoning and procedures contributed by each worker on this subject.

The physiological and clinical aspects of pernicious anemia have been reviewed by Minot and Strauss in an earlier volume (91a), and will be presented only briefly here.

## II. DEFINITIONS

Pernicious anemia, a member of a not too closely knit family of anemias, was recognized as a clinical entity in 1849 by Addison (1) and named about twenty years later by Biermer. Because of this dual sponsorship, it is often referred to as Addison-Biermer disease. Because anemia may refer to a deficiency in red blood cells, in hemoglobin content of the cells, or both, a classification of anemias on an etiological basis has been found useful:

- (1) Anemias mainly due to loss of blood from hemorrhage.
- (2) Those resulting from an excessive erythrocyte destruction.
- (3) Those due to defective erythrocyte formation.

Pernicious anemia belongs to group No. (3). It is believed that in pernicious anemia the early large forms of the red cells, the megaloblasts, tend to divide but not to mature, so that, whereas the blood cells normally range in size from 6 to 9  $\mu$  with an average value of 7.5, in pernicious anemia the peak of the distribution curve of corpuscle size (Price-Jones curve) lies between 8.25 and 9  $\mu$ .

In pernicious anemia, three organ systems are chiefly involved (115).

### 1. *Gastro-Intestinal Tract*

#### 1. Glossitis.

2. *Achylia gastrica*, associated with an atrophy of the gastric mucosa and the usual absence of both pepsin and hydrochloric acid from the gastric secretions. It is generally felt that such a condition is a necessary finding in order to justify the diagnosis of pernicious anemia. Indeed, achylia has been reported to have existed ten, thirteen, and even twenty years before the anemia developed. In such cases, although with treatment the symptoms of anemia may be lessened, the achylia persists unaltered. The frequency of the association of achlorhydria with pernicious anemia has led to the views that:

(a) Without achlorhydria, the real cause of the disease (*e.g.*, toxic products, micro-organisms), could not be effective; or

(b) Achlorhydria in itself is the main causative factor.

In evaluating such hypotheses, however, we should remember that many people with *achylia gastrica* never develop pernicious anemia.

3. Short periods of diarrhea as well as occasional constipation.

### 2. *Blood*

(1) Reduced number of red blood cells.

(2) High color index.

(3) Megalocytosis (megalocytes are red blood cells with a diameter of 12-25  $\mu$ ).

(4) Poikilocytosis and anisocytosis.

(5) Leukopenia.

(6) Reduction in number of platelets.

(7) High serum bilirubin.

(8) Increased excretion of urobilin in stools and urine.

(9) Marked deposits of hemosiderin in the liver, spleen, and kidneys.

(10) Red cells slightly less fragile than normal.

(11) In the bone marrow, replacement of fatty marrow of the long bones with the red marrow very rich in megaloblasts.

### 3. *Central Nervous System*

Changes in the central nervous system may occur with or without actual anemia (100, 115), although they are always associated with *achylia gastrica*. The essential lesion is a primary degeneration of the myelin sheath with no glia reaction, most often affecting the posterior columns and the pyramidal tracts.

## III. EARLY THERAPY

Up to 1926, various treatments were tried with dubious success. These were based for the great part on the hypothesis that pernicious anemia is a

disease of infectious, toxic, or neoplastic nature and that the change in blood picture is caused by pathological hemolysis. In 1925, Elders emphasized the similarity between tropical sprue and pernicious anemia (31). He was of the opinion that sprue is a deficiency disease, having treated it successfully since 1916 by dietary control. His recommended diet consisted "of underdone meat up to 1 kg. or more, with 1½ to 2 l. of raw milk with aqua calcis 5 times a day 15 cc., 15 or 30 cc. of codliver oil, and a few oranges or 400 g. of strawberries a day." He reported a case of pernicious anemia greatly improved on this diet. He thus suggested that pernicious anemia is also a deficiency disease.

Koessler, Maurer, and Loughlin in 1926 (70) reported that the diet is of great importance in the etiology and treatment of pernicious anemia, and on the basis of this observation, formulated a vitamin deficiency theory of pernicious and other severe anemias. From clinical observations, the study of the histories of pernicious anemia patients, especially in relation to diet, and experimental work with rats, they considered that adequate treatment must include the replenishment of the body with vitamins through a proper dietary management. For this purpose, they recommended a great number of food-stuffs rich especially in vitamins A, the B-complex and C. It is of interest that Koessler, *et al.* strongly urged the inclusion of liver and kidney in the diet of the pernicious anemia patient. In 1927, Koessler and Maurer (69) reported on the further success of the treatment of pernicious anemia through the administration of a high caloric diet rich in vitamins.

When Cohn, *et al.* in 1927 (15, 16) prepared an active liver extract free from vitamins, Koessler and Maurer (69) argued that although liver undoubtedly contains a hematopoietic-stimulating substance, liver alone in the absence of ample vitamins will not lead to permanent lasting results.

Although later work has shown that vitamins A and C are not concerned in pernicious anemia, the suggestion of Koessler and associates that pernicious anemia is a deficiency disease was fundamentally sound.

Meanwhile in 1925, Whipple and associates (121), investigating the treatment of severe secondary anemia of dogs brought on by repeated hemorrhages, observed the favorable influence exerted by the feeding of beef liver. They concluded that the "body stores in liver parent substances which are used in the construction of hemoglobin and red cells" (121). In a later paper they said: "It is probable that in human beings food factors will be found more efficient in the control of simple anemia than iron or other drugs. Even in complex anemias (human pernicious anemia. . .) food factors deserve serious consideration in the clinical management of the blood condition" (122). Their suggested applicability of liver therapy in hemorrhagic anemia to pernicious anemia needed to be put to clinical test.

## IV. WHOLE LIVER THERAPY

The effectiveness of whole liver therapy in pernicious anemia was reported by Minot and Murphy (90, 91) in 1926. These investigators reported the prompt and distinct improvement in a large number of pernicious anemia patients on a diet in which liver was an important constituent. Within two to eight days of such treatment, there occurs an increase in reticulocytes of the circulating blood, reaching a maximum on the third to tenth day and subsequently returning to the lower original level. With continued liver therapy, this reticulocyte response is followed by a rise in hemoglobin and total red cell count, with a return to an approximately normal blood picture in about two months. A sudden improvement in mental outlook and a rapid gain in weight are also quite characteristic of satisfactory response to treatment.

In the clinical assay of a preparation, the increase in reticulocytes is often looked upon as proof of activity. However, there is frequently a lack of correlation between the degree of reticulocyte response and the rapidity of increase of red blood cells. A good reticulocyte response usually, but not invariably foretells a good erythrocyte increase. On the other hand, a poor reticulocyte response is often followed by a satisfactory increase in erythrocytes. For this reason, less attention is paid, at present, to the reticulocyte rise as a measure of the effectiveness of a tested material. Observation of the magnitude of the reticulocyte rise gives merely an indication of *presence* of potency of a given material, but not of *degree* of potency.

Inasmuch as the chief difficulty in the work on the purification of active materials has been the relative scarcity of pernicious anemia patients, many worthy attempts have been made to develop an animal assay; the guinea pig, dog, cat, pigeon, swine, monkey, and rabbit have all been tried without definite success if any. Creskoff and Fritz Hugh have covered this subject admirably in their review of standardization and assay of liver extracts (20). At any rate the clinical assay still is the only reliable way of following the fractionation procedures.

Investigations of the chemical nature of the active principle in liver were begun soon after Minot and Murphy had disclosed that such a substance or substances must exist. Whether the antianemic factor occurs in normal liver in a preformed, active state, as we tend to believe, or whether it is a result of post-mortem autolysis is still disputable. Although Herron and McEllroy observed an increase in the hematopoietic activity of liver upon autolysis (50), Castle and Strauss were unable to demonstrate such an increase in activity (11). However, Roth (96) concluded that liver itself does not contain measurable quantities of immediately effective anti-pernicious principle. He attributed the activity of liver and liver extracts to post-mortem transformation of Hämogen (corresponding to Castle's extrinsic factor) into a potent form by a specific cell enzyme. Of interest

is the report of Wilkinson, Klein, and Ashford (128) that the antipernicious anemia principle (whether in active form or as an inactive precursor), is present in the liver of patients with aplastic anemia in amounts at least equal to those in normal human liver, whereas it was absent from the liver of a patient with untreated pernicious anemia who died of pulmonary embolus.

In this review, we shall be concerned only with the substance or substances in liver which exert a beneficial influence in cases of pernicious anemia. We shall not discuss Castle's *intrinsic* factor, the material secreted normally for the most part by the pyloric glands of the stomach, and by Brunner's glands, or his *extrinsic* factor present in such food materials as meat, eggs, yeast, and liver (7, 9, 10, 12, 13, 103). The claim of Klein and Wilkinson (68) that the active thermostable principle of liver can be synthesized *in vitro* by incubating beef muscle and hog stomach could not be confirmed by Castle (8). We shall not concern ourselves with the anti-anemic substance reported to be present in normal urine (129, 114) (25, 113, 78, 63) nor shall we attempt to decide whether the antianemic principles found in the kidney (86), lungs (52), brain, salivary glands, saliva (109) pancreas, and other organs are the same chemically as that which occur in liver. We shall but mention that a report has been made of "a new therapy of pernicious anemia" with a spinach extract, a report however, not founded on experiment (93). Nor shall we attempt to review the interesting observations made by Massa and Zolezzi (81, 82, 83) and Mermod and Dock (88) on the use of congo red in pernicious anemia.

## V. FRACTIONATION OF LIVER BY COHN, MINOT, AND ASSOCIATES

### 1. *Procedures Employed in Separation*

In 1927, Cohn, Minot, and their collaborators (15) attacked the problem of the isolation of the substance or substances in liver effective in the treatment of pernicious anemia. Their general procedure was to divide raw minced beef liver into various portions such as:

- (1) Water-soluble and insoluble,
- (2) Protein and non-protein,
- (3) Lipoid and non-lipoid,
- (4) Dialyzable and non-dialyzable components.

They followed the course of the active principle by noting the presence or absence of a reticulocyte response after the administration of an extract to a pernicious anemia patient during relapse. At first, the material was given orally, but later in the work, it was administered intravenously. In case of negative clinical results, the responsiveness of the patient was tested with liver or liver extracts of proved potency.

The procedures employed in the separation of the several fractions are presented schematically below (Table I). [The first part of the diagram is

TABLE I

Inactive Fractions	Raw Minced Liver brought to pH 9.0	Active Fractions
Insoluble Residue (A)		Water-Soluble Fractions brought to pH 5.0
Protein Precipitate (B) (acid-precipitable proteins)		Water-Soluble Fractions heated to 70°C.
Heat-Coagulable Proteins (C)		Water-Soluble Fractions (D) extracted with ether
Ether-Soluble Fractions (EE) [Removes 2% of solids of (D)]		Non-Ether-Soluble Fractions (E) extracted with strong alcohol
Alcohol-Soluble Fractions (F) [Removes about 30% of solids of (D)]		Alcohol-Precipitable Fractions (G) dialyzed
Dialyzed Fractions (H)		Dialyzate (I) treated with silicic acid gel (pH 5)
Extractives Adsorbed by Silicic Gel (J)		Filtrate (K) extracted with n-butyl alcohol
Residues of n-Butyl Alcohol Extraction (M)		Fractions (L) precipitated with basic lead acetate
Lead-Precipitable Fractions (N)		Filtrate (O) precipitated with phosphotungstic acid
Filtrate from Phosphotungstate (Q)		Precipitate (P) Phosphotungstates treated with 90% acetone
Acetone-Soluble Phosphotungstates (S)		Acetone-Insoluble Phosphotungstates (R)
		Starting with precipitate (P) regenerated and treated with 95% alcohol
Insoluble Residue (peptones, proteoses, polypeptides)		Extract concentrated Intravenous Extract "I. E."
		"I. E." dissolved in 90% alcohol, added equal volume of ether
Filtrate (contains tryptophan, tyrosine)		Precipitate. Treated with 1 volume H <sub>2</sub> O: 9 vol. alcohol: 4 vol. ether
Precipitate (large number of substances giving diazo test)		Filtrate. Treated with 1 volume H <sub>2</sub> O: 12 vol. alcohol: 6 vol. ether
Filtrate (extracts phosphorus-containing substances)		Precipitate (Z). Treated with 11 vol. alc.: 6 vol. ether: 1 vol. H <sub>2</sub> O
		Precipitate (this is the fraction that had hitherto been precipitated by HgSO <sub>4</sub> in acid solution). Treated with picric acid
		Precipitate (extremely active). (Effective in 140 mg. dose.)

reproduced from papers (15) and (16), the rest from discussions in the authors' later papers (17, 18, 19). The letters designating the different fractions are those used by the authors.]

Residue (A) contains, among other things, connective tissue and water-insoluble proteins, fats, and carbohydrates. (B) is the copious, characteristic liver protein, the isoelectric point of which is in the neighborhood of pH 5. (A) and (B), both separately and together, showed no clinical activity. Heat coagulation at 70° C. removed most of the proteins, largely the albumins and globulins of the blood. Extract (D) concentrated under diminished pressure at 60° C., when given in the diet of four pernicious anemia patients produced a prompt increase of reticulocytes and a subsequent increase of red blood cells. It was free from blood sugar reducing substances. When (D) was concentrated at higher temperatures (*i.e.*, on a water bath), it still showed some activity but less than that of liver pulp, indicating, perhaps, that the active principle had been largely destroyed during the prolonged concentration at high temperature. Separation into (F) and (G) was obtained by pouring a concentrated aqueous solution of (D) into an amount of absolute alcohol which would result in a final alcohol concentration of approximately 95 per cent by volume. Precipitate (G) which had been freed from such lipids as are soluble in ether, acetone, and strong cold alcohol, and from all but a trace of protein, sulfur, and iron, proved effective when fed daily in amounts of 9–14 g. This is equivalent to approximately 200–400 g. of whole liver. (It was later found that fraction (G) can safely be given parenterally. When thus administered, its effectiveness is considerably greater—4 g. given parenterally being as effective as 100 g. by mouth.)

Thus, in 1927, Cohn, Minot, *et al.* (15) reported a relatively purified product (G), neither lipoidal nor protein in nature, the partial analysis of which follows:

Non-protein, non-ammonia N	6.3–8.8 per cent
Phosphorus	1.7–3.5 per cent
Ash	16.0–20.8 per cent

It may be interesting to mention at this point a few facts concerning the clinical activity of two of these fractions. Fraction (D) was fed to a patient, 9.45 g. of solid material for the first 10 days, then 18.9 g. for the following 5 days. The reticulocytes ranged from 2.1 per cent the first day to 0.3 per cent the fifth day, 16.0 per cent the tenth day and 2.8 per cent the fifteenth. The erythrocyte concentration in millions per cubic millimeter showed no appreciable rise during the 15 days (1.24–1.32). On another patient, the red cells rose from 2.75 millions to 4.48 millions per cmm. in 36 days (16). Clinical evidence of this sort indicated the presence of the factor in (D).

Fraction (G) was fed to another patient in amounts ranging from 7.24 to 14.48 g. for 22 days. The reticulocyte percentage rose from 0.1 before feeding to 41.4 on the fifth day of feeding and then fell to 0.4 by the 22nd day. The concentration of erythrocytes in millions per cubic millimeter rose from 0.55 to 2.94 in the 22 days. This would indicate good activity.

In 1928 (16), Cohn and associates reported that inasmuch as the active principle appeared in neither fraction (A) nor (B), it seemed advisable to eliminate the preliminary extraction: The minced liver, therefore, was brought immediately to pH 5, in the cold, thus inhibiting the hydrolysis of carbohydrates and proteins. They also found that most, if not all, of the material effective in pernicious anemia was dissolved by treating fraction (G) with 70 per cent alcohol. In this alcohol mixture, a large amount of inert material remained undissolved. Thus, to purify fraction (G) further, fraction (D) was dissolved in 70 per cent alcohol; the filtrate and washings were concentrated *in vacuo* and poured into absolute alcohol to make a 95 per cent mixture. The hygroscopic precipitate obtained was still a relatively crude product from the chemical standpoint, although a very satisfactory one therapeutically when administered orally.

The observation that the substance is effective when given by mouth suggested that the dimensions of the molecule are not such as to interfere with its absorption. Indeed, after electrodialysis, the residue (H) proved inactive when fed to a patient.

## 2. Lack of Relation between Fraction (G) and Known Vitamins

In view of Koessler's work, the question of the relation of the active material from the liver to the known vitamins was of some concern. The steps through which the extract had been carried with a retention of potency precluded the presence of those vitamins that are lipid-soluble. Laqueur and Münch in an examination of seven preparations all prepared according to the methods developed by Cohn and his associates found no evidence of vitamin A (75). The properties of vitamin C (*e.g.*, its instability) excluded it as a possibility. This conclusion has been verified by Koser, who in 1936 reported that the activity of antianemic preparations could not be ascribed to vitamin C (72). When "vitamin B" was concentrated by adsorption on silicic acid gel at pH 5 (79), the antianemic principle was left in the filtrate (16). Additional evidence against its possible vitamin B character was that the feeding of large amounts of food, rich in anti-neuritic and pellagra preventing factors, to pernicious anemia patients led to no reticulocyte response. In this work, yeast cake, dried aqueous yeast extract and vitamin extract were employed. The investigators declared, however, that further research along these lines was needed (16).



### 3. Evidence against Carbohydrate Character

Slightly more than one half the constituents of fraction (G) were precipitated by basic lead acetate at pH 8, the active principle remaining in the filtrate (O). This preparation (O) was free of carbohydrates as judged by the Molisch test. Thus, proteins, fats, and carbohydrates had been eliminated from the raw beef liver without a noteworthy loss in activity.

The authors stated that: "Moreover, many organic acids form lead salts and would have passed into fraction (N). There remained chiefly neutral or predominantly basic molecules, and in large part, the inorganic constituents of the liver that had passed into fraction (G)" (16).

Analyses showed that whereas the purer fractions contained less phosphorus, they were richer in nitrogen. Thus, the per cent of phosphorus and nitrogen in fractions (O), (G) and (D) were:

	<i>Per cent of solids</i>		
	(O)	(G)	(D)
Total P	1.02-2.97	2.14-4.38	2.61-4.95
Total N	11.66-15.36	10.15-13.81	6.92-16.36

The inference drawn from such results was that "the active principle is either a nitrogenous base or a polypeptide" (16).

### 4. Evidence Suggesting Nitrogenous Base Character

Nitrogenous bases and some polypeptides are precipitated by phosphotungstic acid. The phosphotungstates of the nitrogenous bases are far more soluble in acetone-water mixtures than are the phosphotungstates of the polypeptides. Treatment of fraction (O) with phosphotungstic acid precipitated the active principle. That portion of the precipitated phosphotungstates which dissolved readily in 90 per cent acetone-water mixtures proved active. Such evidence pointed to the nitrogenous base, rather than the polypeptide, character of the material.

When preparation (S) was brought to an acid reaction, a precipitate formed. Also, after regenerating the acetone-insoluble phosphotungstate (R), a precipitate appeared at acid reaction (maximal precipitation occurred at pH 1.5-2). This acid precipitable material, which appeared to be similar in fractions (R) and (S), showed activity. Upon analysis, it was found to have 7.11 per cent N, 1.10 per cent P and 62 per cent ash; on an ash free basis, its nitrogen is 18.7 per cent. Cohn and associates considered that this precipitate consisted of phosphotungstates of nitrogenous substances, due, perhaps, to the incomplete removal of phosphotungstic acid in regeneration.

At about the same time, Laqueur and Münch analyzed various liver extracts prepared by the methods of Cohn and associates from beef and pig liver (75, 76) with the following results:

Total N	7-8	per cent
Urea	2-3	per cent
NH <sub>2</sub> -N	2-3	per cent
Lipoid	0.3-0.5	per cent
S	0.7-0.8	per cent
P	0.6-1.0	per cent

In 1929, Cohn, Minot, and McMeekin reported that although only slightly soluble in 95 per cent alcohol, the active principle was dissolved in such a mixture, following regeneration of the precipitated phosphotungstates (P) (17). The residue, containing proteoses, peptones, and polypeptides, proved inactive. The antianemic factor was not precipitated by trichloroacetic acid nor by tungstic oxide; hydrolysis produced no increase in amino N; the biuret test was negative. Hence, on the basis of the previously mentioned inference (page 246), the active principle seemed to be a nitrogenous base rather than a peptide.

#### 5. Preparation and Properties of Intravenous Material

Concentration of the alcoholic extract of (P) led Cohn and associates to the precipitation of active material free from the depressor substances of previous extracts. This active precipitate, designated I.E., was successfully injected intravenously, since neither proteins nor blood pressure-reducing substances were present. No purine bases appeared in I.E.; no material was precipitated by flavianic acid from neutral solution. In highly purified solutions, heavy metals appeared to destroy activity.

In 1929, Cohn and associates reported that I.E. had the following properties (17):

- (1) Soluble in H<sub>2</sub>O, alcohol, phenol, glycerol, formamide, acetic acid.
- (2) Combined with acetic acid, it dissolved in chloroform and carbon tetrachloride.
- (3) Fraction I.E. is extracted by pyridine or by butyl alcohol.

In 1930, Cohn, McMeekin, Minot (18) reported the further purification of fraction I.E. by extraction with various percentage mixtures of H<sub>2</sub>O, alcohol and ether as shown in our diagrammatic representation of their procedure (Table I). Preparation (Z) exhibited *negative* biuret, Molisch, Pettenkoffer, Millon, nitroprusside, and diacetyl reactions. From such tests as well as other reactions we have already discussed, they concluded that the active principle must be free from proteins, carbohydrate, lipoids, tryptophane, tyrosine, arginine, cystine, creatinine, iron, sulfur and phosphorus. (The phosphorus-containing substances present in earlier products had been removed in the process of preparing (Z) by treatment with 1 vol. H<sub>2</sub>O: 12 vol. alcohol: 6 vol. ether. This precipitated the active principle, whereas the phosphorus-containing substances remained in

solution.) Fraction (Z) gave no precipitate with trichloroacetic, picric or picrolonic acids nor with flavianic acid in acid solution. It contained substances which were precipitated by phosphotungstic acid, mercuric sulfate in acid solution, gold chloride, platinic chloride and silver nitrate.

#### 6. Evidence against $\alpha$ -Amino Acid Character

When (Z) was dissolved in a small volume of 20 per cent alcohol and placed in the cold, a white crystalline precipitate was obtained. This product was redissolved and reprecipitated from 20 per cent alcohol containing 5 per cent ether. The resulting material proved active. However, repeated crystallization yielded a crystalline product, all the nitrogen of which was  $\alpha$ -amino nitrogen, and which contained carboxyl groups equal in number to the amino groups. This substance, the nitrogen content and solubility of which suggested isoleucine, proved inert.

Cohn and associates had noted that mercuric sulfate gave a precipitate with active fractions, but that both the precipitates and the filtrates regenerated after precipitation by mercuric sulfate always proved inactive. It was believed that the heavy metal precipitated the active substance, but also inactivated it in the process. A method for separating the isoleucine from the active portion (mercuric-sulfate-precipitable) was then sought. Advantage was taken of the fact that the isoleucine is less soluble than the mercuric-sulfate-precipitable portion in the presence of small amounts of alcohol at low temperatures. Thus, treatment of solution (Z) with 11 vol. of alcohol, 6 vol. of ether, and 1 vol. of water resulted in the precipitation of an active material containing no appreciable amount of  $\alpha$ -amino nitrogen.

As the purification of the active principle proceeded, interference of contaminating substances on its solubility was diminished. Thus, picric acid failed to precipitate the active principle until after the removal of the  $\alpha$ -amino acids.

Solubility and electrophoretic studies together with its precipitation by Ba and Ca in 90 per cent alcohol, suggested the presence of a carboxyl group in the molecule (19). The same investigators suggested also that perhaps they were not dealing with an  $\alpha$ -amino acid, but with an  $\omega$ -amino or imino acid.

#### 7. Summary of Studies by Cohn and Collaborators

From such studies, Cohn, McMeekin, and Minot in 1930 (18, 19) believed that the active principle was a nitrogenous base, the nitrogen in which exists as in a secondary or tertiary amine. The low nitrogen content (10.8 per cent) appeared to exclude purine or pyrimidine bases, but not ring compounds of the pyrrole or pyridine types. It seemed unlikely that it was a pyrrole because it gave no pine-splinter test characteristic of certain pyrroles.

In summary, one of their tables is reproduced to illustrate the outstanding progress they made in the method of fractionation, purification, and understanding of the nature of the active principle effective in pernicious anemia (19) (Table II).

#### VI. EARLY WORK BY WEST AND ASSOCIATES

West and Nichols in 1928 (120), starting with a commercial extract (fraction G of Cohn), and following the methods of further purification as developed by Cohn—*i.e.*, use of basic lead acetate, phosphotungstic acid and silver, obtained a product the analyses of which they reported as:

N	12-14 per cent
NH <sub>2</sub> -N	20 per cent of total N
NH <sub>2</sub> -N after acid hydrolysis	40 per cent of total N
P	None
S	Faintest trace
Fe	None

They also reported that their material had the following qualitative properties:

Biuret	Positive
Diazo	Positive
Hopkins-Cole	Weakly positive
Naphthol test for arginine	Positive
Polariscope	Weak levorotation

In 1930, West and Howe (116, 117) reported the preparation of a product which on intravenous injection of 680 mgm. caused a reticulocyte rise from 3 per cent to 40 per cent, with a red cell rise from 1.4 to 2.3 millions per cubic mm. This material, obtained as an amorphous precipitate, was acid to methyl red and showed a levorotation of about  $-15$  degrees.

<i>Analysis.</i> C	46.6 per cent
H	6.9 per cent
N	10.6 per cent
O	35.8 per cent
S	None
P	None
NH <sub>2</sub> -N	Trace
NH <sub>2</sub> -N after hydrolysis	50 per cent of the N

It yielded amorphous barium and green copper salts which were soluble in H<sub>2</sub>O and precipitable by alcohol. Other properties which characterized the material were:

With phosphotungstic acid	Heavy precipitate
With HgSO <sub>4</sub>	Moderate crystalline precipitate
With alcoholic PtCl <sub>4</sub>	Moderate crystalline precipitate

TABLE II

Date	Method of Fractionation	Knowledge of Active Principle	Amount given per day, g.
1926	—	Contained in liver	200-300
1927	Extract with water at protein isoelectric point	Water-soluble	
	Extract proteins coagulated by heat or alcohol	Not a protein	16
	Precipitate with abs. alcohol (not extracted with ether)	Not a lipid	10
	Not pptd. with lead acetate	Not a carbohydrate (Molisch negative)	8
1928	Pptd. with phosphotungstic acid (pptd. with mercuric acetate) (pptd. with tannic acid: Krogh)	Nitrogenous base, peptide, or proteose?	6
	Not pptd. with $\text{Na}_2\text{SO}_4$ : West extract with 95% alcohol	Not a proteose, nor a peptide (Biuret negative)	4
1929	Precipitate with 95% alcohol	Not a depressor base (injected intravenously)	1.5*
	Precipitate with $\text{H}_2\text{O}$ :alcohol:ether 1 : 9 : 10	Millon negative	1.0
	Extract with $\text{H}_2\text{O}$ :alcohol:ether 1 : 9 : 4	Diazo weak	0.7
1930	Ppt. with $\text{H}_2\text{O}$ :alcohol:ether 1 : 12 : 6	Not a phosphorus compound	0.3
	Remove leucine crystals at 0°C. Ppt. at pH 3 with $\text{H}_2\text{O}$ :alc.:ether 1 : 11 : 5	Not an $\alpha$ -amino acid	0.08
	Precipitate with picric acid	Ninhydrin negative	0.025

\* This and all subsequent fractions have been injected intravenously. In order to compare the very different procedures involved, the amount necessary to produce a maximal reticulocyte response, though injected in one or two days, is recorded as though given over a period of six days.

With ammoniacal $\text{AgNO}_3$	Slight precipitate
With picric, flavianic, picrolonic acids	No precipitate
With $\text{AuCl}_3$	No precipitate
From alcohol with thallous hydroxide	Amorphous precipitate
No reduction of $\text{KMnO}_4$ in acid, slight in alkaline solution	
Diazo	Negative
Molisch	Negative

When distilled with soda lime, vapors were obtained which gave a strong pine-splinter reaction. Among the hydrolytic products, there was found a substance which gave a green copper salt and was precipitable by phosphotungstic acid.

Since no reaction was obtained with the usual reagents which precipitate basic materials, except with phosphotungstic and phosphomolybdic acids, they concluded that their best fraction was rich in a nitrogenous body with acid properties.

Later in the same year, these same investigators reported the preparation of the quinine salt of this acid in a finely crystalline form which retained its activity after three recrystallizations. They suggested  $\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}_2 \cdot \text{C}_{10}\text{H}_{18}\text{N}_2\text{O}_6$  as the formula of this salt.

Among the products of hydrolysis of the active material by 10 per cent  $\text{H}_2\text{SO}_4$ , they were able to identify  $\beta$ -hydroxyglutamic acid and later  $\gamma$ -hydroxyproline. They were of the opinion that their material was a peptide or diketopiperazine which, on hydrolysis, gave  $\beta$ -hydroxyglutamic acid and  $\gamma$ -hydroxyproline.

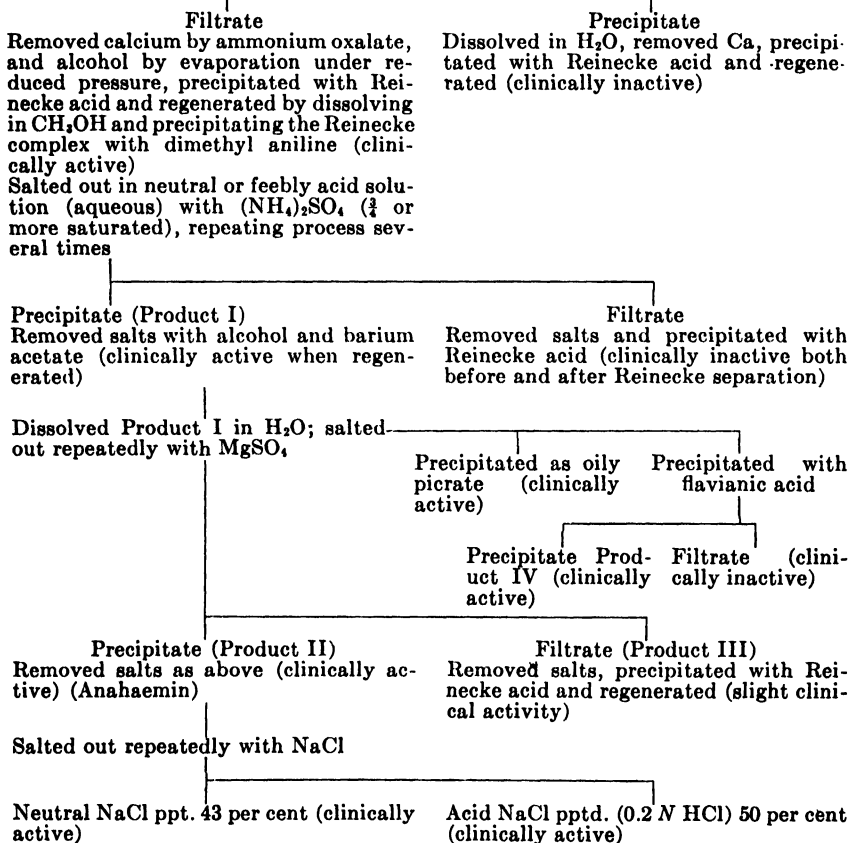
In 1931 (118), they questioned the correctness of their previous conclusion that the quinine salt was active. Although they obtained some positive clinical results, there were other cases which proved completely negative and which subsequently responded typically to therapy with whole liver.

#### VII. LATER WORK BY DAKIN, WEST, AND ASSOCIATES

In 1935, Dakin and West reported a different method of fractionating Cohn's fraction G (22). A preliminary purification was obtained by treating the extract with calcium acetate or chloride in 75–80 per cent alcoholic solution, the active material appearing in the filtrate. The active material was precipitable by Reinecke salt in acid solution. In the regeneration of such a precipitate, the bulk of the Reinecke acid was separated from a weak alcoholic solution of the active material as the sparingly soluble salt of a tertiary base—*e.g.*, dimethyl aniline. The small amount of additional unprecipitable Reinecke acid was removed with solvents such as amyl alcohol which did not remove the active material. These procedures were carried out at virtually neutral reaction. The steps involved in the preparation described are diagrammed in Table III.

Very little chemical separation was effected by treatment of Product I with picric acid. However, flavianic acid precipitated 65-75 per cent of Product I as an oily, somewhat unstable substance, which contained the active principle and thus constituted definite progress in the course of

TABLE III  
Commercial Liver Extract  
treated with calcium acetate in 75% alcohol



purification. Unfortunately, the difficulty of regenerating the active material from the precipitate and the fact that flavianic acid is a very imperfect precipitant for the substance, unless the latter is in a rather pure form, made such a step inefficient in the process of fractionation.

It was found that about 75 per cent of Product I was precipitated by saturation with  $\text{MgSO}_4$  in aqueous solution. That a chemical separation is

accomplished here was shown by the fact that a repetition of the process on the  $\text{MgSO}_4$  precipitate brought down about 97 per cent of the material. This was Product II which proved active, 80 mg. giving a maximal response.

The authors explained the slight residual activity of the filtrates following  $\text{MgSO}_4$  precipitation as due to one (or perhaps both) of two factors:

- (1) Incomplete separation of  $\text{MgSO}_4$  precipitable material;
- (2) Other active products.

Further fractionation was effected with NaCl. 43 per cent of Product II separated from a neutral saturated solution of NaCl, while an additional 50 per cent precipitated from the filtrate on adding HCl to a concentration of 0.2 N. These two preparations were very similar to each other; both were active, although the neutral NaCl precipitate was possibly more active than the acid NaCl precipitate.

All these products obtained as powders by precipitation with alcohol-ether and later absolute alcohol were very soluble in dilute alcohol.

The hydrolysis of preparations such as Product II yielded: trace of histidine, 14 per cent arginine, 5 per cent lysine, 15 per cent leucine, 10-14 per cent hydroxyproline, a monoaminodicarboxylic acid fraction including aspartic and glutamic acids, 41-45 per cent, 5 per cent glycine, and 15 per cent aminohexose. Complete absence of pyrimidine and purine bases, pentoses, and desoxyglucose was reported.

*Analysis* (of such preparations, Product II).

C	50.0-51.4 per cent
H	7.0- 7.2 per cent
N	15.2-15.4 per cent
$\text{NH}_2\text{-N}$	0.4- 0.5 per cent
$\text{NH}_2\text{-N}$ after hydrolysis	10.6-10.8 per cent

In their 1935 paper, Dakin and West gave prominence to the possibility of the importance in the pernicious anemia problem of the aminohexose found among the products of hydrolysis of their active preparations. Absence of sugar groupings, other than those of an aminohexose type, was indicated by negative reactions with phloroglucinol, naphtho-resorcinol and Tillmans-Philippi tests. They were of the opinion that the aminohexose (more specifically glucosamine) was present in an anhydride type linkage with the amino acids.

The fact that the preparation was not precipitated by ferrocyanic or rufanic acid but was brought down by tannic acid led Dakin and West to allocate the compound to the peptone rather than the albumose group. On the other hand, its easy separation with  $(\text{NH}_4)_2\text{SO}_4$  might be regarded as indicative of an albumose character. However, it is known that some synthetic peptides of only three or four amino acids are salted out by ammonium sulfate (22).

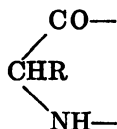


These investigators, employing the method of titratable acidity, suggested that the molecular weight of their active product was a multiple of 1430.

A study of the action of various enzymes (22) on their material revealed that:

- (1) Pepsin in 0.1 *N* HCl caused a slow hydrolysis, 8–9 per cent increase of amino nitrogen in relation to total nitrogen in 14 days. Material when treated with pepsin for 5 days still showed activity when tried on a single case.
- (2) Pancreatic juice had no hydrolytic action on the substance.
- (3) Erepsin brought about a slow but almost complete hydrolysis.

The fact that normal sodium hydroxide in 24 hours hydrolyzed the substance with complete racemization of the arginine, lysine, and leucine groups seemed to indicate that the  $\alpha$ -amino and adjacent carboxyl groups were internally bound as



Later work by Dakin, Ungley, and West in 1936 on the separation of their product into glucosamine-rich and glucosamine-poor fractions convinced them that the aminohexose was not an integral part of the active principle (23). They obtained, at this time, very active preparations completely free from glucosamine. The steps in the preparation of such a product have been represented diagrammatically in Table IV. The preliminary purification with alcoholic calcium acetate was replaced by twice salting out with saturated  $(\text{NH}_4)_2\text{SO}_4$ . See Table IV on opposite page.

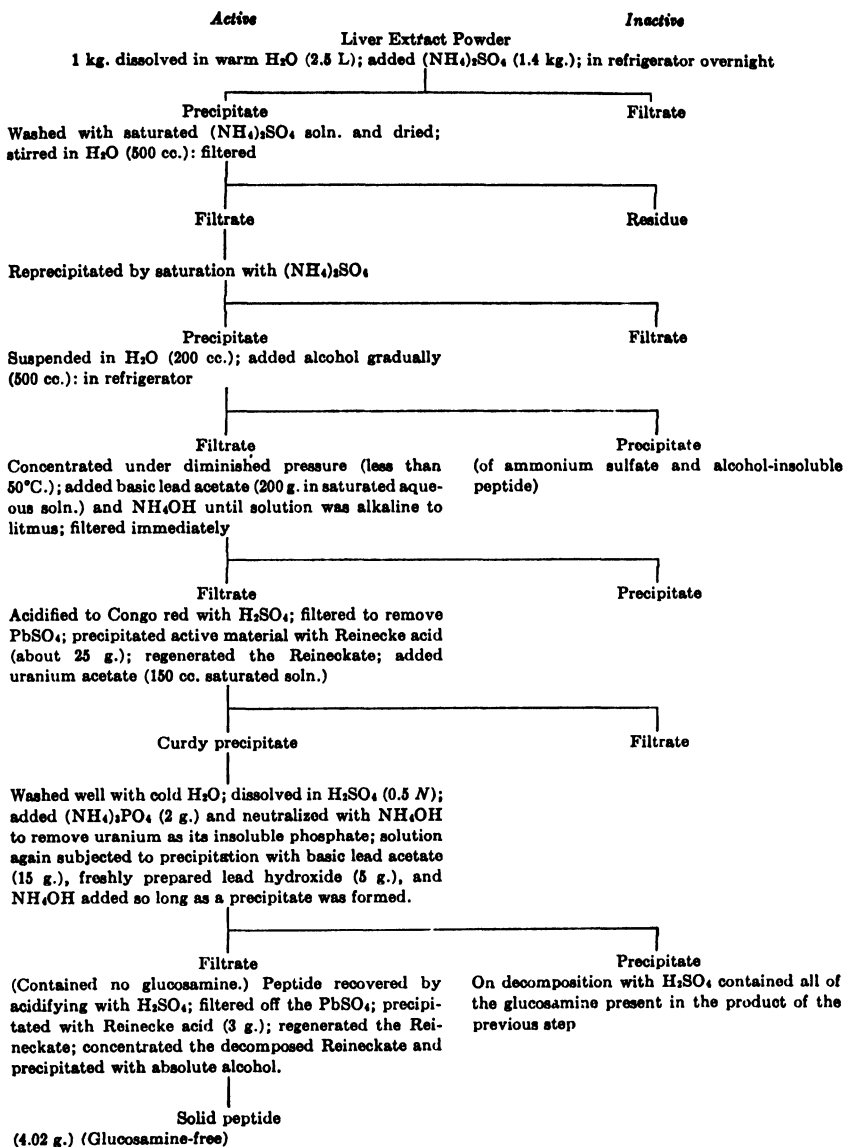
*Analysis* (of such a preparation).

C	46.8–48.1 per cent
H	6.6– 6.8 per cent
N	15.9–16.5 per cent
Amide N—	0.5 per cent
NH <sub>2</sub> -N	0– 0.2 per cent
NH <sub>2</sub> -N after hydrolysis	10–10.4 per cent
Specific rotation $[\alpha]_D^{25}$	–112° to –133°

In view of the absence of glucosamine and lysine in this product, they concluded they were not essential components of the active material.

Since the active material was not precipitated by rufanic, ferrocyanic, and metaphosphoric acids, nor trichloroacetic acid in low concentration, nor by copper acetate and ferric sulfate, they believed that the active material was of a peptide rather than an albumose nature.

TABLE IV



Hydrolysis yielded chiefly: arginine, glycine (9.5–10.4 per cent), leucine, aspartic acid, hydroxyproline, and perhaps proline. There was also indication of a dicarboxylic acid easily soluble in H<sub>2</sub>O and giving a very soluble

copper salt precipitable by alcohol. Possibly, this was hydroxyglutamic acid. By dialysis experiments through membranes of known pore diameter, they tentatively assigned a molecular weight between 2000–5000.

Whereas sodium hydroxide inactivated the product, treatment with  $\text{NH}_4\text{OH}$  (1 *N*) for 2 days produced very little change in optical activity. From such a result, Dakin, Ungley and West (23) concluded that exposure to  $\text{NH}_4\text{OH}$  during the process of isolation probably was without deleterious effects.

It may be of interest to compare the main conclusions arrived at by Cohn, and his associates, and by Dakin, West and their associates in their latest published works. Cohn, McMeekin, Minot concluded that "the very small amount of  $\alpha$ -amino N . . . suggests that we are not dealing with an  $\alpha$ -amino acid but with an  $\omega$ -amino or imino acid. The low nitrogen content appears to exclude purine or pyrimidin bases but not ring compounds of pyrrole or pyridine types, though the purest fractions do not give the pine-splinter test characteristic of certain pyrroles," (19). The conclusion reached by Dakin, Ungley, and West, as stated in 1936, was "that the hemotopoietic substance in liver is, or is associated with, a peptide, possessing many but by no means all of the properties of an albumose" (23).

In 1942 (119), West and Moore further separated the active material obtained by Dakin, Ungley and West into two separate fractions by electrophoretic methods. "Two main components have been observed, one of slow mobility and the other of greater mobility and positively charged at pH 4.0. The charge is reversed at about pH 7.0." The slow component was active and the fast practically inert. A single 40 mg. injection of the slow component prepared from a commercial extract was given to a patient and was followed in seven days by a rise of reticulocytes from 1.4 to 16.2 per cent and a rise of the red cells from 2 to 2.3 million. The amounts of the fast and slow moving components in the above preparations were divided equally.

## VIII. WORK OF LALAND, KLEM, STRANDELL, AND ASSOCIATES

### 1. *Chemical Procedures*

Meanwhile work on the problem was being carried out by other groups of investigators. One of those whose work has resulted in an important advance toward the isolation of the active material is the Scandinavian group. In 1935, Strandell reported that he had obtained successful clinical results with material of high potency (101). This was followed in 1936 by a paper by Strandell, Poulsson, and Schartum-Hansen (102). In 1936, Laland and Klem (74) reported the method of preparation of the active material.

Their procedure of fractionation (101) is founded chiefly on the observation that phenol may advantageously be employed for the elution of material after adsorption on charcoal—especially of nitrogenous compounds. Their attempt to use this reagent in the concentration of the hematopoietic principle of liver has met with success. The steps they employed in carrying out the separation may be shown diagrammatically in Table V. We have retained the terminology employed in the authors' reports (101).

At this stage, the solubility of fraction BBa was tested in methyl and ethyl alcohol. It was found to be more easily soluble in the former than the latter. Thus, all of the fraction which is soluble in ethyl alcohol can also be dissolved in methyl alcohol, whereas, the reverse is not true. Taking advantage of the differential solubility of the active fraction in the two alcohols as well as its insolubility in ether, Laland and Klem were able further to fractionate BBa. The steps are shown in Table VI.

In this way, four fractions were obtained, all of which are clinically active. They reported the distribution of the antianemic substance in the various fractions as follows:

(1) BBa <sup>1</sup>	0.0002 g. dry substance from 100 g. liver
(2) BBa <sup>2b</sup>	0.0002 g. dry substance from 100 g. liver
(3) BBa <sup>3a</sup>	no data given
(4) BBa <sup>3ab</sup>	0.0003 g. dry substance from 100 g. liver

Inasmuch as this treatment seemed to divide the active principle among different fractions rather than accomplish a definite chemical separation, Laland and Klem abandoned this procedure as a method of further purification.

Dakin and West in 1935 found it possible to salt out the active substance by  $\frac{3}{4}$  to complete saturation with  $(\text{NH}_4)_2\text{SO}_4$ . Laland and Klem also found that such treatment of their fractions yielded active material, although they report that the antianemic principle was not precipitated quantitatively even by full saturation with ammonium sulfate.

By a series of steps, the exact details of which have not yet been reported, Laland and Klem have obtained an active fraction, "BBaBFu.s.E.," 0.00035 g. of which in dry state correspond to 100 g. of liver. This material is a bright reddish-yellow, non-crystalline acidic substance, easily soluble in water, partly soluble in alcohol, and insoluble in ether. They reported absorption bands in two regions of the ultraviolet range—namely at 250–265  $\text{m}\mu$  and 345–350  $\text{m}\mu$ . Phosphotungstic acid and a series of heavy metal salts precipitated the antianemic material from an aqueous solution. The ninhydrin reaction was negative; Tollen's orcin test positive. After hydrolysis, amino-nitrogen as well as acidic and basic amino acids have been detected.

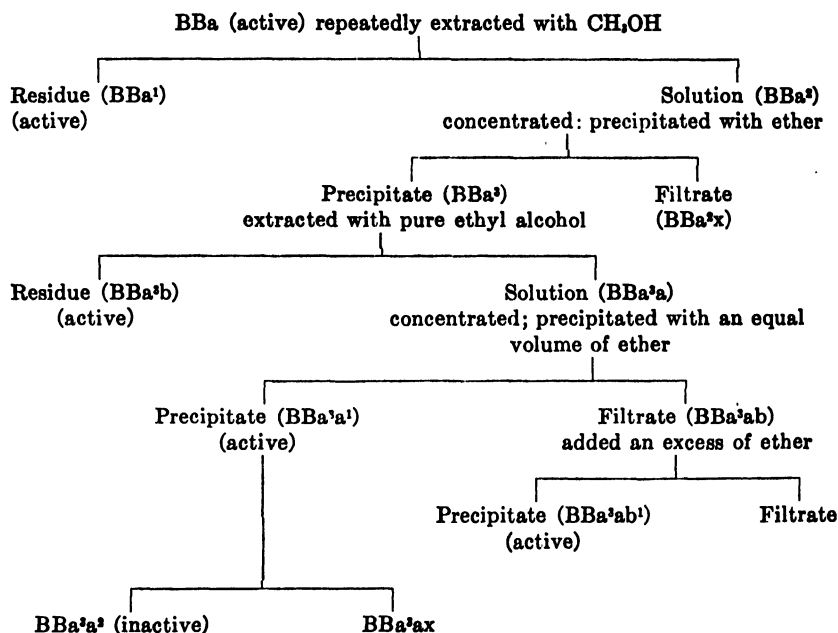
TABLE V

<i>Active</i>		<i>Inactive</i>
Fresh hashed liver		
Extracted with H <sub>2</sub> O with addition of acetone to 50 pct. by volume (tissue H <sub>2</sub> O inclusive)		
Extract I strongly concentrated		Residue
Concentrate (II) filtered		
Filtrate (III) freed from protein—different methods used: precipitation by metal sols or gentle heat coagulation with the addition of acid		
Protein-free filtrate (IV) (Pernami I) free from albumin but not from salts. 3–4 gm. dry matter from original 100 g. liver shaken with phenol—this extract (IV) gives off the active substance quantitatively to phenol along with other substances. Add ether and H <sub>2</sub> O to the phenol solution—the antianemic substance can be quantitatively shaken into the water layer		
Fraction VI (clinically very potent) (Heptomin I—dark) 0.27 g. dry matter from 100 g. liver. Treat with active “coal” (charcoal)		
Coal adsorbate extracted by phenol and regenerated by shaking with ether and H <sub>2</sub> O		Filtrate
Fraction VIII (fully active) (Heptomin II—Yellow) 0.028 g. dry matter from 100 g. liver com- bined the phenol extraction with extraction with H <sub>2</sub> O containing phenol		
Fraction B 0.001 g. dry matter from 100 g. liver addi- tional adsorption on active charcoal and combined extraction with phenol water		Fraction A 0.019 g. dry substance from 100 g. liver (inactive)
Fraction BB 0.001 g. dry matter from 100 g. liver evap- orated to dryness, dissolved in glacial acetic acid and precipitated with excess ether		
Fraction BBa (nearly colorless) 0.001 g. dry substance from 100 g. liver		

	N	C	H	S	Ash
<i>Analysis.</i>	13.33	53.64	6.85	0.74	2.05

Laland and Klem also reported the non-identity of the strongly yellow-green fluorescent liver flavine with the antianemic principle, a confirmation of an earlier finding of Stare and Thompson that liver flavin given intramuscularly to five pernicious anemia patients did not give rise to a hematopoietic response (99).

TABLE VI



## 2. Clinical Assays of Activity

Strandell and associates have reported on the clinical activity of many of the fractions just discussed. In 1935, Strandell (101) demonstrated that 53 mg. of Heptomin II (corresponding to about 200 g. liver) in aqueous solution when injected intragluteally showed remarkable antianemic activity. Of interest is his report of a patient suffering from a pronounced degeneration of the spinal cord; here Heptomin II brought about a considerable improvement not only of the blood picture, but also of the neurological symptoms when given in doses of 56 and 84 mg.

Fraction B proved active when injected in two doses of 10 mg. each. BBa<sup>1</sup> and BBa<sup>3b</sup> each showed high activity when dissolved in water (2 cc.)

in the quantity of 1 mg. dry material, (corresponding to 500 g. of liver), and administered intragluteally in two doses. 2.7 mg. of dry BBa<sup>a</sup>ab<sup>1</sup> (corresponding to 800 g. of liver) in aqueous solution (2 cc.) was sufficient to start remission when administered intragluteally in one dose.

Strandell, Poulsson, Schartum-Hansen (102) reported that 4 cc. of a solution containing 0.7 mg. of fraction BBaBFu.s.E and corresponding to 200 g. of liver, when injected parenterally gave a good hematopoietic response. The reproduction of one of their charts (Table VII) may serve to illustrate the potency of this preparation.<sup>1</sup>

TABLE VII

Case No.	Date 1936	Dose	Reticulocyte	R.B.C.	Hb
		mg.	per cent	millions	per cent
II	April 2	0.7	0.3	0.91	20
	4	0.7	2.2	—	—
	8	—	45.3	2.1	35
	16	—	2.1	2.7	50
	21	0.7	0.3	2.0	35
	23	—	3.2	—	—
	27	—	12.6	2.04	40
	May 3	—	0.8	—	—
	6	—	—	2.45	55

### 3. Application of Laland and Klem Procedures to Dakin and West Material

Ungley reported in 1936 (112) the results of an investigation of the clinical activity of an active Dakin and West fraction of liver, further purified by the methods of Laland and Klem. The greater purification was obtained by employing phenol and methyl alcohol for fractionation. Dakin and West's fraction, Product II (see page 252), was dissolved in 2.5 parts of 95 per cent phenol. Anhydrous methyl alcohol was then added slowly to effect fractional precipitation. The first fraction was discarded and the later fraction retained. A slight buff colored product was obtained (217A) of which 14.3 per cent was nitrogen.

Fraction 217A was injected intramuscularly. While the response to 25 mg. 217A was often small, 50 and 75 mg. gave satisfactory results, comparable

<sup>1</sup> It should also be mentioned that in 1935, Strandell (102) reported that he obtained favorable clinical results with certain fractions of liver, the chemical preparation of which has not been reported. A preparation, called fraction E, when dissolved in 2 cc. H<sub>2</sub>O to the extent of 0.020 g. (corresponding to 100 g. of liver) proved active. Fraction BPhSol of which 0.0086 g. in dry condition can be obtained from 100 g. of liver elicited a typical reticulocyte but only a very slight erythrocyte response—e.g., a case was given 10 injections of 0.0086 g. each in a period of four weeks. BF (0.0099 g. dry substance from 100 g. liver) showed splendid results.

to those produced by 200 mg. of the original Product II (also known as anahaemin). However, inasmuch as the variations in response to a given dose even in individuals with similar initial red blood cell levels were so great, Ungley thought it advisable to compare the potency of the two products by use of the double reticulocyte response method. Both preparations were administered successively to the same individual. When uniform quantities of potent material were given daily to a pernicious anemia patient in relapse, there was only a single reticulocyte response; but if, after administering daily for 10-14 days amounts of material just sufficient to provoke a submaximal reticulocyte response, the dose was then increased or a more potent product substituted, a secondary reticulocytosis occurred. When the potency of the material given in the second period was equal to or less than that given in the first period, no such secondary response was observed. It was essential that the material given daily in the first period be insufficient to produce a maximal response, otherwise no secondary reticulocytosis could be expected. With such a procedure, 217A proved at least two and one half times as potent as the original preparation. In this way, Ungley has confirmed that the methods of Laland and Klem are valid ones for the purification of the active principle.

#### 4. *Work by Wilkinson*

In 1936, Wilkinson (126) applied Reinecke acid precipitation as employed by Dakin and West to the preparation of active liver extracts, (124, 125, 127). He compared the clinical activity of his liver product with those prepared according to the 1935 method of Dakin and West and that of Laland, Klem, Strandell, *et al.* Dakin and West's material showed maximal reticulocyte response with a dose of 58 to 120 mg. administered intramuscularly. With preparations similar to those used by the Scandinavian workers, Wilkinson obtained maximal response with a total dose of 40 to 80 mg. (68). With his own product, Wilkinson was able to elicit maximal response with total doses of 18 to 36 mg., representing an original amount of 661 to 1332 g. of fresh liver.

#### 5. *Work of Karrer and Associates*

Karrer, Frei, and Fritzsche in 1937 (66) reported that liver preparations which possess full antianemic potency in a singly administered dose of 10-20 mg. contain considerable quantities of phosphorus, the magnitudes of which seem to run parallel with the activity. In their best fractions, they found about 3.8 per cent of this element. Since they also observed pentose and adenine occurring in amounts corresponding to the presence of phosphorus, they concluded that their preparations contain an adenine-nucleotide.



A later paper by Karrer, Frei, and Ringier (67) indicates that phosphorus, pentose and most purine bases are excluded as constituents of the active material. Preparations free from these substances were reported to be active in doses of 8 to 10 mg. Nor did their active material contain flavin, pterin or reducing carbohydrates. The ninhydrin test was positive, before and after hydrolysis, but the biuret was negative or at best only weakly positive.

Analysis (of a highly purified preparation).

C	H	N	S
45.68	6.75	14.63	small amount

Even though these analytical figures are similar to those of proteins and polypeptides, no polypeptides of the usual type were present. After hydrolysis,  $\alpha$ -amino acids were present only in small quantities, if at all. Boiling the hydrolyzed neutral solution with copper carbonate failed to yield the blue coloration characteristic of the  $\alpha$ -amino acids. After 6 N acid hydrolysis, the amino N rose from 0.8% to 9% and after tryptic hydrolysis to 2%. Among the amino acids present were arginine and tyrosine but no phenylalanine, proline, oxyproline, glycine, tryptophan, and histidine. The procedure used by Karrer follows in Table VIII.

These extracts prepared by Karrer were tested by Koller (71). These fractions which were tested at about 2 mg. a day indicated good activity although in some cases the erythrocyte regeneration was slower than in those cases presented by other investigators. His cases are presented in Table IX.

In a short note in 1939, Tschesche and Wolf described the properties of an active material that resembled the preparation of Karrer, *et al.* in that it showed a negative or only slightly positive biuret test, but a positive ninhydrin both before and after hydrolysis (111). Their material, a white powder active in a dose of 40 mg., also had a negative Molisch and a negative Millon test; it was free of flavine, purine, pterine, reducing sugar and phosphoric acid esters.

Analysis. C 50, H 7, N 14.5, S 0.6.

## IX. WORK OF SUBBAROW AND JACOBSON

### 1. The Multiple Factor Hypothesis

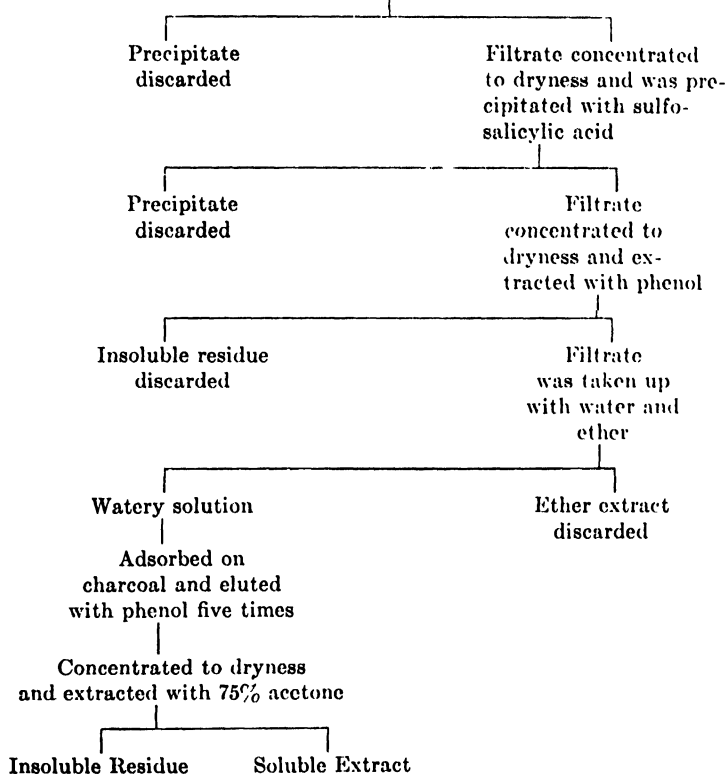
So far there has been presented the efforts of various groups of investigators to isolate the *unique* fraction of liver active in the treatment of pernicious anemia. These investigators worked on the general hypothesis that the active portion is a single chemical entity. The fact that the reports of the chemical properties of their active preparations differed might lead to some doubt that a single substance is involved. The possibility exists

that the activity of material effective in the treatment of pernicious anemia rests upon a certain type of compound or linkage common to more than one substance, as is believed to be the case of some of the vitamins. Then again, one cannot neglect the consideration that in a disease as complex as pernicious anemia, successful therapy may depend upon the inter-

TABLE VIII

*Karrer's Procedure*

Finely ground liver  
was extracted with 50% acetone



action of several factors. It is possible that in order that the full activity of the antianemic principle be exerted, it needs to be supplemented by one or more substances which are themselves inactive.

In 1935, Fiske, SubbaRow, and Jacobson presented results which suggested that the application of liver therapy in pernicious anemia could be achieved most successfully by a combination of two or more substances

TABLE IX

Fraction # Patient #	9c 1	9c 2	9c 3	9c 4	9dA 5	9dB 6	9dG 7	9dP 8	9dS 10	12 11	25a 12	28(a) 15	34(a) 17	35b 18
Total amount of fraction admin- istered	25 mg.	30 mg.	10 mg.	20 mg.	5 mg. 1st day 20 mg. 7th day	30 mg.	20 mg.	60 mg.	20 mg.	26 mg.	20 mg.	15 mg.	20 mg.	18 mg.
R.B.C. at begin- ning, million/ cmm.	1.3	1.37	1.49	1.97	2.17	1.8	1.24	1.3	1.46	1.23	3.0	2.59	1.44	1.48
R.B.C. at end, million/cmm.	2.7	3.5	2.36	2.9	3.3	2.4	1.78	2.4	1.87	2.4	3.37	2.54	1.78	1.76
Reticulocytes at peak	35%	10%	28%	9.6%	12.2%	17.6%	21.3%	25%	29%	22.4%	10%	17.2%	15.6%	26.1%
Day of reticulo- cyte peak	6th	4th	5th	6th	11th	6th	4th	5th	6th	6th	5th	7th	7th	5th
Length of period in days	12	12	15	14	16	15	6	18	10	17	10	13	11	6

(36, 104). This led to the development of a multiple factor hypothesis. A report by Jacobson and SubbaRow in 1937 (61) has indicated that there is (1) a primary, active, hematopoietic factor in liver, and (2) at least three accessory factors, in themselves inactive, but whose presence materially augments the activity of the primary substance. They observed that continued purification of active liver extract, in the absence of significant losses and of destructive procedures, led to a partial loss of therapeutic activity, but that combining these purified preparations of reduced potency with certain inactive fractions, resulted in the restoration of activity.

In the succeeding section, the methods of chemical fractionation and the clinical evidence supporting the hypothesis will be given. The four factors believed to be concerned are:

(A) The primary factor of unknown chemical nature.

Active in amounts of 0.2 to 0.4 mg. per day.

(B) Three accessory factors.

Inactive, singly or in combination.

(1) Fraction A<sup>2</sup>—*l*-tyrosine

(2) Fraction C—probably a complex purine

(3) Fraction F—probably a peptide

Although these authors recognized that the only valid test for therapeutic activity is the sustained successful response of the hematopoietic system of a pernicious anemia patient, they also used the appearance of a reticulocytosis in the guinea pig to aid them in following the presence or absence of activity in the early stages of fractionation.<sup>3</sup> The validity of the guinea pig test, described by Jacobson (58, 59, 60) has been questioned by some investigators [Ågren, Caspersson (2), Goodman, Geiger, Klumpp (47), Lassen, Jacobsen, Nielsen (77), and Bachrach and Fogelson (5) but confirmed by others (Miller and Rhoads (89), Clark and Coene (14), Mermod (87), Jones (64), and Hummel (53)]. Although the reason for the divergence of results in the hands of different workers is not wholly explained, it would appear that the diet of the guinea pigs must be controlled and the amount of blood withdrawn must not exceed 0.1 cc. It has been shown by Jacobson that the accessory factors by themselves give positive reticulocyte responses in the guinea pig but, in the pernicious anemia patient, the primary factor is also required for the initial as well as the complete hematopoietic response. The primary factor is, however, without effect on the guinea pig. For this reason Jacobson and SubbaRow,

<sup>2</sup> The letters designating the fractions of SubbaRow, *et al.* should not be confused with those of Cohn, *et al.*

<sup>3</sup> In their early work, they defined activity of their preparations in terms of guinea pig units, *G.P.U.* The minimal amount of material per kilogram guinea pig, which, after a single intraperitoneal administration, induces a positive reticulocyte response in at least two of three reactive guinea pigs, is termed the Guinea Pig Unit.

in their later work (61), relied upon the sustained increase in the red blood cells of the pernicious anemia patient in evaluating the activity of their preparations.

## 2. Chemical Procedures

In 1935, Subbarow, Jacobson and Fiske reported the isolation of certain fractions using the guinea pig test as a guide and starting with a commercial liver extract (104). The procedure which they used to obtain their fraction A was as follows (Table X).

TABLE X  
*Commercial Preparation*

(328,000 G. P. U. per 100 g. fresh liver)
precipitated with 25 per cent basic lead acetate
↓
Filtrate
regenerated with H <sub>2</sub> S
↓
Solution
removed impurities by adsorption on Lloyd's reagent
↓
Filtrate
precipitated with mercuric acetate
↓
Precipitate
regenerated with H <sub>2</sub> S
↓
Solution
(2.0 mg. total N per 100 g. fresh liver)
(160000 G. P. U. per 100 g. fresh liver)
decolorized with small amount of charcoal and concentrated
↓
Crystalline precipitate (Fraction A)
(48,000 G. P. U. per 100 g. fresh liver)
(1 g. of pure crystalline substance assayed 16,700,000 G. P. U.)
(this material was inactive in pernicious anemia)

The properties of fraction A proved to be those of *l*-tyrosine: readily crysallizable from hot water; melting point, 310–314 °C. on rapid heating;  $[\alpha]_D^{25}$  of  $-6.7^\circ \pm 0.5^\circ$ ; needles in clusters with a greyish tinge; showed a strong ninhydrin and Millon's; reduced Folin-Ciocalteau phenol reagent.

<i>Analysis.</i>	C	H	N	NH <sub>2</sub> N
Calculated for tyrosine	59.64	6.12	7.74	7.74
Found in fraction A	59.66	6.07	7.83	7.8

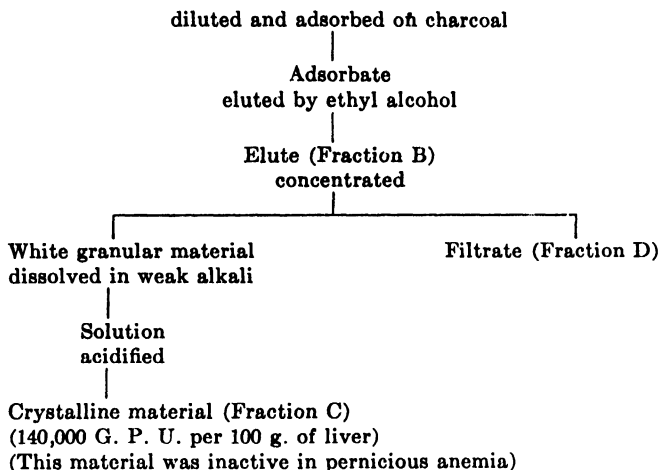
Quantitative estimation by the Millon reagent yielded 95.6 per cent and by the phenol reagent, 97.6 per cent of the respective values given by commercial tyrosine. Confirmatory evidence that fraction A was tyrosine was

the fact that commercial *l*-tyrosine when assayed showed a G.P.U. value identical with that given by crystalline fraction A (104).

The procedure followed in the preparation of fraction C was (Table XI):

From 100 g. of fresh liver, they isolated 11 mg. of fraction C in the form of light yellow crystals. It proved to be non-hygroscopic, difficultly soluble in cold but more soluble in warm water. On heating, it turned brown at 270° C. and decomposed without melting above 400° C. It showed positive xanthine, murexide and diazo tests and reduced the Folin-Marenzi uric

TABLE XI  
*Crude Liver Extract*



acid reagent. They were able to prepare in crystalline form the nitrate, hydrochloride and sulfate of this material.

Analysis of fraction C.

C	H	N	Ash
37.88	3.04	33.07	0.7

From these properties, the conclusion was drawn that the substance is a complex purine, resembling members of the pterine series of Wieland and Schöpf. Later work by SubbaRow indicates that fraction C is composed, for the most part, of xanthine, accompanied by several other difficultly separable substances.<sup>4</sup>

<sup>4</sup> Fraction C, although it crystallizes fairly readily, is found to be an impure mixture. Kohler and M. Tishler (personal communication) showed by (1) perchloric acid precipitation, (2) methylation by diazomethane and the subsequent isolation of caffeine, that it consists mainly of xanthine (about 80-85 per cent). By chromatographic adsorption of fraction C on Florisil column and elution of the column which is strongly fluorescent, 5 per cent xanthopterin was isolated. Xanthopterin has not been tested for reticulogenic activity in guinea pigs and has been found inactive in a patient with pernicious anemia.

In 1936, SubbaRow and associates (105) reported the preparation of an active product by the elution with 65 per cent ethyl alcohol of an aqueous extract of liver after adsorption on chareoal—a method which they had already used in the isolation of fraction C (104) and one which is quite similar to that developed by Kyer at about the same time (73). At this time, they described an improved method of preparing fraction B, a concentrated but impure material, active in pernicious anemia patients (Table XII):

TABLE XII

150 cc. Commercial liver extract (equivalent to Cohn's G)
(3 cc. from 100 g. fresh liver)
(328,000 G. P. U. per 100 g. liver)
dissolved in 1 liter H <sub>2</sub> O
↓
Solution
brought to pH 8 with NaOH, acidified to PH 6 with HCl
added 50 g. norit and stirred 1 hour, filtered
↓
Charcoal + Adsorbate
washed repeatedly with H <sub>2</sub> O till washings colorless
suspended in 1 liter 65 per cent ethyl alcohol, brought to boiling point
stirred 5 minutes and filtered hot. Elution repeated
↓
Combined elutes
concentrated under diminished pressure at 40°C. to 150 cc.
↓
Fraction B
(164,000 G. P. U. per 100 g. fresh liver)

Fraction B, light brown in color, was sterilized by boiling and injected intramuscularly. They reported results on six pernicious anemia patients on whom this preparation was therapeutically active in amounts of approximately 100 mg. of material given over a 10 day period (Table XIII).

In a later report (106), SubbaRow and Jacobson described briefly the properties of another fraction—fraction F, obtained from fraction B—which appeared to be a polypeptide. It yielded crystalline salts with rhodanilic acid and Reinecke salt. The rhodanilate, showing a positive biuret, diazo and glyoxylic but negative Millon's reactions, was precipitated by phosphotungstic, picric, rufanic and tannic acids as well as by ammonium sulfate; the Reineckate, showing a positive ninhydrin and a weak diazo reaction, was precipitated by phosphotungstic and rufanic acids. Fraction F was inactive in the guinea pig as well as the pernicious anemia patient.

# ANTI-PERNICIOUS ANEMIA SUBSTANCES OF LIVER

TABLE XIII

Patient	J. T.	C. H.	F. W.	A. T.	J. D.	C. H.
Date (1935)	9/27	1/16	1/3	10/1	2/20	2/1
Red blood cells in millions per cmm. at beginning of exptl. period	3.51	1.18	2.47	1.07	2.10	1.36
Red blood cells in millions per cmm. at termination of exptl. period	4.07	1.69	2.82	2.42	2.68	2.28
Reticulocyte peak <i>per cent</i>	5.6	10.6	7.8	31.8	11.2	26.6
Length of exptl. period <i>days</i>	10	9	9	10	8	11
Total amt. of fresh liver from which administered extract was derived <i>g.</i>	67	72	88	100	103	200
Total amount of nitrogen administered <i>mg.</i>	8.3	9.4	13.4	12	14	24

## 3. Purification of Primary Factor

Thus far, SubbaRow and associates had isolated various accessory factors which, in themselves, were shown to be of no therapeutic value. Their subsequent work on the purification of the primary factor will now be described (107) (Table XIV). Fractions H and I prepared as described in the table were tested for their activity.

They also prepared fraction I without the use of absorption by Fuller's earth (Table XV).

Fraction I, a white microcrystalline material, was found to be readily soluble in water. Its sulfate, readily soluble in dilute alcohol, but quite insoluble in absolute alcohol, was prepared by acidifying an aqueous solution of fraction I to pH 2.5 with H<sub>2</sub>SO<sub>4</sub> and adding acetone and ether.

*Analysis* (of the sulfate, which decomposes without melting above 290°).

C	H	N	Total S	SO <sub>4</sub>	Ash
41.56	6.74	13.13	4.6	3.4	1.6

Its amino nitrogen was determined after interaction with nitrous acid at the end of five minutes and at the end of 60 minutes:

	Per Cent NH <sub>2</sub> -N 5 min.	60 min
Before hydrolysis	5	14.7
After hydrolysis for 6 hrs. in 2N HCl	75	87

Fraction I reduced the Folin-Ciocalteau phenol reagent and showed a weak diazo, a positive orcin, and negative Sakaguchi, Millon's, glyoxylic acid and biuret. It was precipitated by phosphotungstic acid and partially brought down by Hopkin's reagent. The reineckate and rufianate salts,



although crystalline, proved to be unsuitable for further purification. An aqueous solution of fraction I, exposed to ultraviolet light, exhibited an intense blue fluorescence; its absorption spectrum showed an inflection between 2480 and 2560 Å. SubbaRow and associates pointed out the similarity between the absorption spectra of their fraction I and the material

TABLE XIV

## Fraction D

(150 cc. from 5 kg. liver)

(10–12 mg. total N per 100 g. liver)

acidified to pH 2 with HCl; added 16 g.

English Fuller's earth; stirred mechanically for 30 min. at room temp.; the ppt. was filtered and washed once with 50 cc. H<sub>2</sub>O

## Filtrate + Washings

added 10 volumes 95% ethyl alcohol and 10 volumes ether; mixture left in cold room 24–36 hours; filtered

## Precipitate (Fraction H)

(20 mg. from 100 g. of liver—12–13% N)

dissolved in 50 cc. H<sub>2</sub>O; brought to pH 3 with 10 N H<sub>2</sub>SO<sub>4</sub>—caused a crystalline ppt. to settle, consisting mainly of CaSO<sub>4</sub>

## Filtrate

added to 40 cc. of H<sub>2</sub>O containing 1 g. Reinecke salt at 40°; in cold room for 24 hours—brought down a crystalline ppt.; filtered

## Precipitate

washed once with 50 cc. ice cold H<sub>2</sub>O; suspended in 300 cc. 0.03 N H<sub>2</sub>SO<sub>4</sub> at 30–35° and the Reinecke acid was removed by repeated extraction with a 500 cc. mixture of equal vols. of amyl alcohol and ether; concentrated *in vacuo* to a volume of 25 cc.

## Concentrate

added 10 vols. acetone and 10 vols. ether; in cold room 48 hours

## Microcrystalline precipitate (Fraction I)

(yield of 100 mg.—*i.e.*, 2 mg. from 100 g. of fresh liver)

(active in pernicious anemia patients)

of Laland and Klem. Data showing the response of patients both to fraction I and the less pure fraction H are shown in Table XVI.

Fraction I, regenerated from the rufianate, was also tested for its therapeutic activity on two patients. The resulting hematopoietic responses were similar to the responses to fraction I described in Table XVI.<sup>5</sup>

<sup>5</sup> In 1933, SubbaRow, Jacobson, and Hartfall (108) reported that "the chemical properties of the primary factor suggest that it is a pyridine derivative." They also reported that in addition to the three accessory factors already reported, they had identified tryptophane and guanosine which seemed to possess some ability to enhance the hematopoietic response, initiated by the primary factor.

Since the methods already described for the isolation of the primary factor gave considerable trouble in their adaption to larger scale procedures, SubbaRow, Jacobson, and F. Ablondi tried to use other methods in the isolation of the active material.

Fraction B, equivalent to 1 ton of liver, was taken up in 16 liters of water and was precipitated at pH 1.5 with 1100 g. of Reinecke salt. This mixture was kept at 0° C. for 12 to 18 hours after which time the precipitate was filtered and washed with ice cold water. It was then taken up in 50% acetone. The Reinecke acid was removed by addition of AgNO<sub>3</sub> and excess silver then removed with NaCl. After removal of acetone in vacuo, the pH

TABLE XV

Fraction D (1 liter)

added 7 liters 95% ethyl alcohol at room temp. for five hours

Filtrate

added 3 liters ethyl alcohol and 10 liters  
ether; in cold room 48 hours

Precipitate

(contains no Fraction I)

Precipitate (Fraction E)

dissolved in 500 cc. H<sub>2</sub>O; added 200 cc. of  
5% solution of rhodanilic acid in CH<sub>3</sub>OH;  
in cold room 48 hours; filtered

Crystalline precipitate

freed of rhodanilic acid by means of pyridine  
with subsequent removal of pyridine by ether

Regenerated rhodanilate solution

precipitated by Reinecke salt and the Reineckate  
regenerated (as in other process of preparing Fraction I)

Fraction I

(yield about the same as in other method)

of this aqueous solution was adjusted to pH 5.0 and adsorbed with 3.0 kg. of charcoal. The charcoal adsorbate was eluted a second time with 13 l. of 50% phenol. The phenol of this second elution was removed with ether and the aqueous phase concentrated in vacuo to a small volume. It was then precipitated with acetone ether. This precipitate yielded 26 g. of solid which contained 4.56 g. of total N per ton of liver (not corrected for ash).

This acetone-ether-insoluble portion when administered to four patients in a daily amount of 3.4 mg. in addition to 6.4 mg. of the previously mentioned accessory factors, gave on the 10th day an average erythrocyte response of from 1.8 million R.B.C. per cmm. to 2.5 million R.B.C. per cmm. with appropriate reticulocyte response (62).

In collaboration with N. Bohonos, material obtained from one ton of liver was dissolved in water and after adjustment of pH to 1.5 was reprecipitated with Reinecke salt. The Reineckate precipitate was extracted with water at 60° C., and this solution was freed of Reinecke acid by extraction with amyl alcohol and ether. This aqueous portion contained 6.6 g. of solid material and its total N was 1.3 g. per 1 ton of liver (not corrected for ash).

The hot-water-soluble Reineckate fraction, when administered in a daily amount of 0.3 mg. in addition to 6.2 mg. of the accessory factors in a case of an initial R.B.C. level of 1.1 millions per cmm., resulted in a maximum reticulocyte response of 35% on the fifth day and at the end of ten days the R.B.C. level was 2.5 million per cmm.

TABLE XVI  
*The Therapeutic Activity of Fraction H and Fraction I*

Fraction No.	H	H	H	H	I	I
Patient No.	16	15	21	22	23	13
Total amount of fraction administered, mg.	60	20	20	20	7, 6	4
R.B.C. at beginning, millions per cmm.	1.05	1.28	1.15	0.88	2.41	1.40
R.B.C. at end, millions per cmm.	2.42	1.93	1.80	1.60	3.10	2.35
Reticulocytes at peak, per cent	31.0	24.4	30.2	24.6	6.6	12.0
Reticulocyte peak, day	6th	7th	5th	5th	4th	5th
Length of period, days	18	8	8	8	10	10

Dr. Benjamin Alexander of the Beth Israel Hospital in Boston, Massachusetts also gave this fraction to a patient in a daily amount of 0.4 mg. without the addition of accessory factors. The initial R.B.C. level was 1.0 million per cmm. A maximum reticulocyte response of 12.4% was obtained on the 6th day and on the 8th day the R.B.C. was found to be 1.6 million per cmm. (personal communication).

When an equivalent of 100 lbs. of this hot water Reineckate soluble fraction was adsorbed with 50 g. of permutite at pH 6.0, the filtrate was completely active and showed 35 mg. of total solids from the 100 lb. equivalent.

This permutite filtrate was also tested by Dr. Benjamin Alexander in a patient with an initial R.B.C. level of 1.4 million. In a daily dosage of 0.035 mg., without accessory factors added, a maximum reticulocyte response of 11.4% was obtained on the 6th day and the final R.B.C. level of 1.9 million was reached on the 10th day (personal communication). The isolation of the active fraction is summarized in Table XVII.

TABLE XVII

Fraction B
2000 lbs. in 16 l. H <sub>2</sub> O
acidified to pH 1.5 with H <sub>2</sub> SO <sub>4</sub>
Added 1100 Reinecke salt, and kept at 0° for 12 to 18 hrs. The ppt. was washed with ice-cold water and dissolved in 50% acetone
↓
50% Acetone solution
Removed Reinecke acid with AgNO <sub>3</sub> and excess Ag with NaCl.—Removed acetone in vacuo and adjusted to pH 5.0
↓
Aqueous pH 5.0 solution adsorbed with charcoal
↓
Charcoal
Charcoal eluted with 34 l. of 5% phenol which was discarded and then 13 l. of 50% phenol
↓
50% Phenol elute
Removed phenol with Et <sub>2</sub> O and concentrated aqueous solution to small volume
↓
Concentrate
Precipitated with acetone-ether
↓
Precipitate
Dissolved in small volume of water and precipitated at pH 1.5 with Reinecke solution
↓
Precipitate
Reinecke ppt. extracted with hot water at 60°C.
↓
60°C. Reinecke solution
Removed Reinecke with amyl alcohol and ether
↓
Aqueous solution
Yield of 2000 lbs. of liver Total solids—6600 mg. Total N—1300 mg.
↓
Adsorbed with 1000 g. of permutite at pH 6.0 and filtered
↓
Filtrate
Yield from 1 Ton of liver 700 mg. (Total solids)

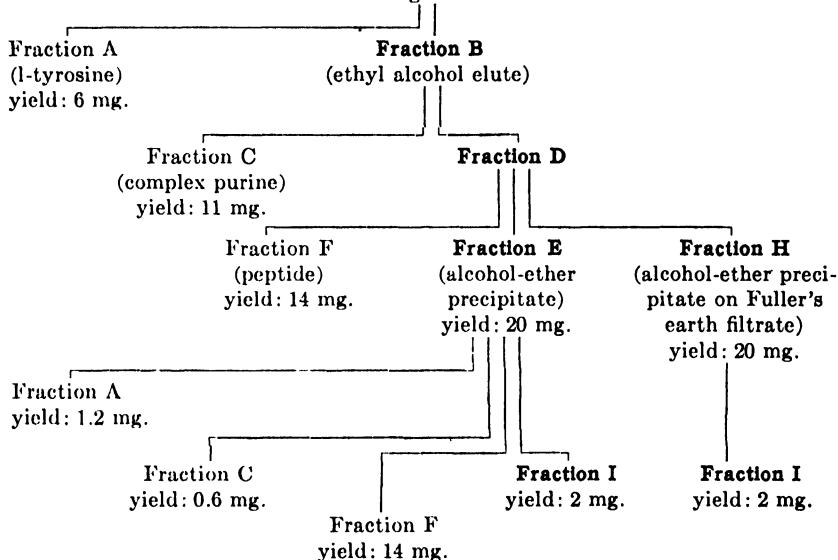
#### 4. Clinical Evidence for Multiple Factor Hypothesis

Chemical procedures used in the isolation from liver of various fractions of importance in pernicious anemia, as carried out by SubbaRow, *et al.*,

are shown in Table XVIII. The fractions containing the primary factor are represented in heavy type.

In 1937, Jacobson and SubbaRow reported the interrelation of these fractions in the treatment of pernicious anemia (61). Their general procedure was to observe the effect on both the reticulocyte and red blood cell response of administering the purified preparations to patients in various combinations and doses. As sources of the primary factor, they used fractions E and H. (E contains F and very small quantities of A and C, whereas

TABLE XVIII  
Crude Liver Extract  
(corresponds to fraction G of Cohn)  
from 100 g. fresh liver



H is completely devoid of A, C, and F.) In most of the experiments, they used crystalline commercial *l*-tyrosine as fraction A.

In the *absence* of the three accessory factors, the effect of either fraction E or H alone upon erythrocyte production was moderate. When fractions A, C and F were administered as well, a satisfactory erythrocyte response was obtained. Data taken from their paper illustrating this point are shown in Fig. 1. The administration of a combination of primary and accessory factors resulted in a rate of erythrocyte production closely approximating that produced by the intramuscular administration of an amount of commercial liver extract containing equivalent quantities of the primary and accessory factors.

The erythrocyte response following the administration of large amounts of the primary factor (H) *without* the three accessory factors was not as great as the average response to smaller amounts of the primary factor when given together with the accessory factors. This is shown in Fig. 1.

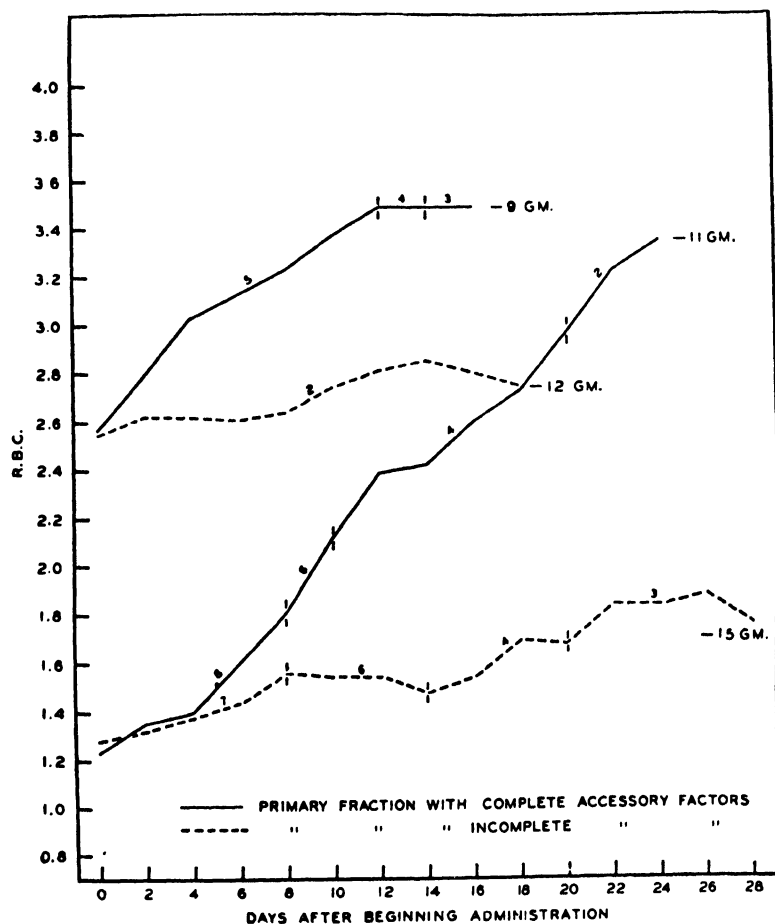


Fig. 1

Averages of Erythrocyte Regeneration Curves at Two Different Initial Erythrocyte Levels, Following the Administration of the Primary Factor with and without a Complete Supplement of Accessory Factors

The number of patients studied in each period is shown by the small figures above each curve. The quantity at the end of each curve denotes the calculated average daily amount of primary fraction administered, expressed in terms of amount of fresh liver from which the extract was derived.

The relative supplementary action of each of the accessory factors was also studied. Their evidence for the importance of fraction A (*l*-tyrosine) was as follows: the administration of an adequate amount of primary factor together with fractions C and F, but with an insufficient quantity of fraction A, was followed in two patients by no erythrocyte response; the addition of a suitable amount of A (*i.e.*, amount derived from 20 g. of liver administered on alternate days) induced a good response as well as general clinical improvement.

Their evidence for the importance of fraction C was based on the following results. The continuous administration, over a ten day period, of primary factor, as fraction E, together with fraction A but with minimal amounts of fraction C (fraction E contained sufficient fraction F), induced only slight reticulocyte and erythrocyte responses. An increase in the amount of fraction C, however, was followed by a satisfactory blood response as seen in Fig. 2.

The accessory action of fraction F was indicated by observations of the same nature. Fractions H, A, and C, in the complete absence of F, induced a rise in erythrocytes of only 0.5 million during a period of 28 days, whereas with H, A, C and F, satisfactory responses were induced. On the other hand, neither fractions A, C, or F individually or together induced significant reticulocyte or erythrocyte responses in patients who subsequently reacted to the administration of crude liver extracts.

Jacobson and Subbarow compared the parenteral and oral administration of tyrosine as an accessory factor (A) on one patient. Six milligrams of *l*-tyrosine (corresponding to fraction A from 100 g. liver) when injected intramuscularly was active as an accessory factor, in spite of the fact that the patient, who was the subject of this investigation, derived several grams of tyrosine daily from ingested protein. No gross evidence of defective protein digestion was observed in the patient. During the first period of 20 days, fractions E (from 20 g. of liver) and C (from 20 g. of liver) were administered on alternate days (E contained F); in addition, 100 mg. commercial *l*-tyrosine were given orally every day. No change occurred in the clinical condition of the patient, and there was only a slight rise in erythrocytes. During the first ten days of the following period, in addition to the same basic fractions of E and C, 1.2 mg. tyrosine (A—from 20 g. liver) was injected intramuscularly on alternate days. Clinical improvement and a significant erythrocyte response ensued. Nineteen days following the last treatment, the erythrocytes had fallen to a lower level; at this point, the same basic fractions of E and C were administered as well as 1.0 g. of tyrosine daily for 10 days by mouth. A prompt erythrocyte response was elicited.

Jacobson and Subbarow have interpreted these results as indicating that there may have been defective absorption of the orally administered

tyrosine, abnormal destruction in the intestine, or faulty utilization after absorption. The possibility of abnormal protein digestion was not excluded however.

From the above results, Jacobson and SubbaRow believe that the primary factor when administered alone exerts only a moderate therapeutic

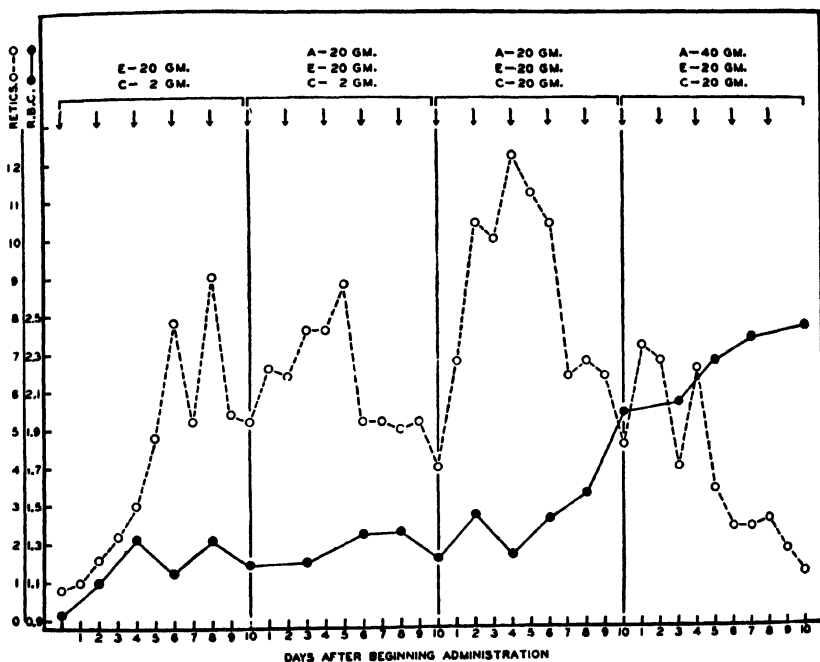


Fig. 2

#### Fraction C as an Accessory Hematopoietic Factor

The quantities above each period refer to the amounts of fractions injected on the days denoted by each arrow. Throughout the four periods Fraction E—20 mg. was administered on alternate days. During the first two periods Fraction C—2 mg. was administered on alternate days. There were only slight reticulocyte responses in the first period in the absence of fraction A and no further erythrocyte rise in the second period after addition of Fraction A—20 g. on alternate days. The increase of Fraction C to —20 g. in the third period resulted in the greatest rise of reticulocytes and satisfactory rise of erythrocytes, continuing in the fourth period.

effect, and, for maximum response, the primary factor must be supplemented by the accessory factors which, in themselves, individually and collectively, are inert. Against the objection that the satisfactory therapeutic activity of the four fractions together might depend upon additive effects of a substance common to all is the fact that A, C, F were separately



and together administered in amounts sufficiently great so that had there been contamination with the primary factor, a response would have been expected. Indeed, these investigators believe that the fractions A, C and F may not constitute all the accessory factors present in crude liver extract. In their later work they also listed guanosine and histidine as accessory factors to induce a complete and sustained clinical and haematopoietic response (62).

#### X. OTHER EVIDENCE FOR MORE THAN ONE FACTOR

Evidence reported by others which suggests the presence of more than one substance in liver extract of complementary therapeutic importance in pernicious anemia will now be described. Experiments by Eisler, Hammarsten and Theorell (29) have indicated that liver extract may be separated into two portions, one of which leads to reticulocytosis, the other, when administered with the first, to erythropoiesis as well. Using cataphoresis, they obtained evidence of two active principles in liver preparations. Substance R, between pH 4.5 and 6.6, migrated toward the positive pole; E, toward the negative pole (not the fraction E of Subbarow, *et al.*). Substance R caused reticulocytosis, but not an increase of erythrocytes. On the other hand, substance E, when given alone, had no measureable effect on either erythropoiesis or reticulocytosis. However, R and E administered together induced a reticulocytosis followed by a strong erythropoiesis. They also reported that R can be replaced by definite chemical agents which in themselves effect a reticulocyte response, such as the protein-free tetra-sodium salt of thymonucleic acid.

Wichels and Hofer in 1934 (123) came to a similar conclusion in respect to the separation of a "reticulocyte response" factor from a general "curative" factor in liver. Healthy people were used as test subjects. Ingestion of raw liver in doses above 75 g. per day caused an increase of reticulocytes in 12 cases. In about one-half of these responsive cases, an increase of eosinophilic leucocytes in the peripheral blood also occurred. The substance eliciting the response, called by these investigators *bone marrow stimulant* (Knochenmark-reizmittel), proved to be heat labile. Administration (usually intramuscularly) of large doses of four commercial liver extracts of proved activity (Hepatrat, Heprakton, Campolon and Pernaemyl) effected in general no reticulocyte response in normal individuals. From such results, Wichels and Hofer felt justified in assuming two antianemic factors in liver, each working fundamentally in a different direction. One, which "cures" pernicious anemia, can be extracted from liver and is concentrated in the known liver preparations. The other, absent or present only in small amounts in the commercial preparations, increases the reticulocyte level of peripheral blood in normal individuals.

An obvious objection to their conclusion is that if they divorce from the pernicious-anemia "curing" factor the ability to elicit a reticulocyte response, it is difficult to explain the reticulocyte increase which does occur in the treatment of pernicious anemia patients with such preparations as those to which they attribute only that factor. It is also doubtful whether a normal person can be used to test for any component of the antianemic principle.

Mazza and Penati (84, 85) have isolated active materials containing, they believe, a nucleotide, a polypeptide, and a pterine. Their steps of fractionation are summarized in Table XIX.

Their substance C which they believe is of pterine nature showed maximal absorption at 2535 Å and 2490 Å.

Substance D contained 3% ash (traces of iron and copper); its microanalysis showed 50.1% C, 7.6% H, and 12.4% N. It also contained phosphorus (about 3%) and sulfur (trace). Ninhydrin and Millon's tests were weakly positive. There were strongly positive tests for histidine and pentose, and a negative test for tryptophan. It showed maximal absorption at 2600 Å and 2650 Å. Of the total nitrogen, 5.8% was amino nitrogen; after acid hydrolysis for 1½ hours amino nitrogen accounted for 68.2% of the total nitrogen. They believe that fraction D consists of a combination of an adenine nucleotide and a polypeptide containing proline, oxyproline, histidine, arginine, hydroxyglutamic acid and a monoamino-monocarboxylic amino acid.

Unfortunately, Mazza and Penati have as yet not reported adequate clinical tests for their material. In the single case which they do report for their purest preparations (C and D), substance D given alone (375 mg. over a period of 8 days) showed no activity. Then in the same case both D (total additional dose of 325 mg.) and C (total dose of 13 mg.) given over a two month period showed only moderate activity, raising the red count from 2.1 million to 3.2 million. From this one case, considering the relatively large amount of material used, their substances are most likely not very pure and thus the chemical properties they have described may be those of contaminating compounds rather than of the antianemic factor.

## XI. ON THE IMPORTANCE OF TRYPTOPHANE AND HISTIDINE

### *Work of Fontès and Thivolle*

From the peptide character of the active material of Dakin and West, and of fraction F of SubbaRow and associates and their evidence for the importance of tyrosine (fraction A), and from the observations of Laland and Klem that amino-acids are among the products of hydrolysis, it would appear that amino acids are of importance in the problem of pernicious

TABLE XIX

Minced pig liver	
Liver juice	
Pressed at 500 atmospheres pressure	
Treated with 96% ethyl alcohol to give a 70% alcohol content	
Dilute $H_2SO_4$ to pH 5	
Boiled for $\frac{1}{2}$ hour	
Precipitate (proteins)	Filtrate
	Concentrated in vacuo to a sirup
	Extracted with trichlorethylene
Extract (lipoids)	Syrupy residue
	Taken up in 8 % alcohol
Precipitate	Filtrate
	Neutralized
Precipitate	Treated with saturated soln. of $CaCl_2$
	Filtrate
Precipitate	Calcium removed by ammonium oxalate
	Filtrate
	Solvent removed in vacuo
	Treated with 5 vols. 98% alcohol
	Precipitate dried in vacuo
	<i>Substance A</i>
	(500 mg. from 500 g. fresh liver)
	(active in total dose of 3 g.)
	Put in aqueous solution
	Treated with soln. of basic lead acetate and $Ba(OH)_2$
	Filtrate
	Pb and Ba removed by $H_2SO_4$
	Filtrate to pH 5
	Charcoal adsorption
	Charcoal and Adsorbate
	Elution with warm (70°) 50% ethyl alcohol at pH 5 (acetic acid)
	Eluate
	Alcohol removed in vacuo
	Charcoal adsorption and elution repeated
	Resulting eluate evaporated to dryness
	<i>Substance B</i>
	(100 mg. from 500 g. liver)
	(Active in total dose of 0.5-1.0 g.)
	(Sky blue fluorescence)
4% Solution <i>Substance B</i> in $H_2O$	4% Solution <i>Substance B</i> in $H_2O$
Precipitated with $H_2SO_4$ and phosphotungstic acid	to pH with HCl
Precipitate	Adsorbed on fuller's earth
	Eluted with 5% pyridine at 70°
	Pyridine Extract
	Evaporated to dryness
	Residue dissolved in 1 N NaOH
	Acidified with HCl
	Filtrate
	Adsorbed on fuller's earth and eluted with 5% pyridine
	Eluate concentrated in vacuo to small volume
	Added $NH_4OH$ , $NH_4Cl$ , and $AgNO_3$
	Precipitate
	Put in solution with 1 N HCl
	Solution adsorbed with fuller's earth which is then eluted with 10% pyridine
	Pyridine eluate evaporated in vacuo to dryness
<i>Substance D</i>	<i>Substance C</i>
(5 mg. from 100 g. liver)	(1 mg. from 5 kg. liver).

anemia therapy. Other investigators have placed emphasis on other amino acids, notably tryptophane and histidine. The use of tryptophane in the treatment of experimental anemia is first credited to Hirasawa (51). In

the early 1930's, Fontès and Thivolle presented evidence in favor of the hematopoietic action of both tryptophane and histidine (38, 39, 40, 41, 42, 43, 44, 45, 46). They regarded the former as the precursor of the tetrapyrrole group of hematin and the latter as the amino acid essential for the formation of globin in the hemoglobin molecule. The body constantly loses the pyrrole nucleus in the form of biliary pigments, urobilin, stercobilin, and porphyrins. However, none of these pigments introduced directly into the organism had hematopoietic action. They concluded that the synthesis of the prosthetic group of hemoglobin is effected from substances other than those of a preformed tetrapyrrole nucleus. Proline, oxyproline and tryptophane have the pyrrole group. The fact that proline and oxyproline are nonessential as well as their presence in tryptophane-free diets which produce anemia in rats seemed to indicate that these amino acids cannot function as precursors of hematin. The hematopoietic action of histidine, constituting 10 per cent of the globin of hemoglobin, is believed to be due to its imidazole nucleus, a nucleus which the organism apparently cannot synthesize. Fontès and Thivolle first showed the hematogenic action of tryptophane and histidine by their observation that subcutaneous injection into normal rabbits and dogs resulted in hyperhemoglobinemia and hypererythrocytosis. This action seemed to depend upon the presence of indol and imidazol nuclei and not to be a function of amino acids in general. Thus, leucine, phenylalanine, and lysine had no blood-forming action (43). Also rats deprived of tryptophane and histidine (abundant proline and oxyproline) developed anemia (44). Alcock in 1933 (3) confirmed the hematopoietic action of tryptophane and histidine in normal rabbits and dogs, but he objected to Fontès and Thivolle's attributing the production of experimental anemia to the lack of tryptophane on the grounds that the diet they employed was inadequate in many respects besides its deficiency in that amino acid. Since he failed to produce an anemia by tryptophane deficiency alone, he was led to doubt that the pyrrole of hematin is derived from tryptophane. However, in 1936 Hamada (48) confirmed the results of Fontès and Thivolle by producing an anemia in rats on a tryptophane-poor diet. Levi has reported that the injection of tryptophane in rabbits with experimentally produced anemia caused a restoration of the red cells and hemoglobin to nearly normal values (80). The work of the French investigators indicated that injection of either tryptophane or histidine separately caused a smaller hematopoiesis than both together, apparently by calling on the body reserve of the other, non-injected one. If tryptophane and histidine do take part in chemical steps leading to the formation of hemoglobin, there should be a proportion at which optimal results are obtained. From the formula of hemoglobin, this ratio was calculated as two weights of histidine per unit weight of tryptophane (41).

Fontès and Thivolle then applied this idea to clinical conditions and

reported that the administration of the two amino acids in leucemia, secondary, splenomegalic, pernicious and neoplastic anemias produced encouraging results. They believed that the digestive disturbances in pernicious anemia may interfere with protein breakdown leading to the production of rather large polypeptides as end products, the absorption of which may be poorly effected. The destruction of tryptophane and histidine by intestinal bacteria, which are relatively increased in pernicious anemia, should also not be neglected (45).

In 1930, they reported the treatment of six cases of pernicious anemia, the results of which prove of great interest (46). The first case was treated with 200 and 400 mg. histidine and 100 and 200 mg. tryptophane, injected subcutaneously or intramuscularly for a month. The patient showed a good remission which was maintained for almost six months without further treatment.

The other cases were treated with similar or even larger doses of tryptophane and histidine. Case 2 showed an initial rapid remission followed by relapse. In case 3, there occurred an initial rapid improvement, a relapse on the 5th day after the commencement of treatment and then a slow gain. Cases 4, 5, 6 did not respond to the therapy.

Thus, these six cases, each showing symptoms justifying a diagnosis of pernicious anemia, responded differently to treatment with tryptophane and histidine; very rapid remission of long duration; slow remission; rapid remission followed by relapse; or complete inactivity. Fontès and Thivolle suggest that perhaps the success of the therapy had a direct relation to the duration of the disease—*i.e.*, most favorable results were obtained in the early stages of pernicious anemia, less favorable responses in the later stages.

Fontès and Thivolle have consistently maintained that the activity of raw liver and of the various liver extracts depends entirely on their content of tryptophane and histidine in the free state. However, Cohn and associates obtained active preparations from which all tryptophane had been removed. Dakin and West reported that hydrolysis of their active product yields a number of amino acids, among which, however, tryptophane and histidine are not included. Aleksandrowicz and Gabryelski (4) found no tryptophane in a commercial preparation of proved activity (Pernaemon). SubbaRow and associates have reported a positive glyoxylic reaction for their accessory fraction F, indicating the presence of an indol ring. It has not been shown, however, whether the constituent of peptide F giving the positive glyoxylic test is tryptophane.

Negative results were obtained by Cuthbertson, Fleming, Stevenson (21), who gave daily injections of 100 mg. tryptophane and 200 mg. histidine dissolved in 10 cc. sterile saline solution to two pernicious anemia patients.

The first case, with a recent history of pernicious anemia, was given injections intravenously for fifteen days, followed by no improvement in blood count, although a moderate reticulocyte response accompanied by a feeling of well-being was observed. Subsequent oral treatment with liver extract effected satisfactory results.

In their second case, one which had previously responded well to both arsenic and liver, Cuthbertson and associates observed that on subcutaneous injection of the amino acids, the clinical condition of the patient became worse. However, after a transfusion, liver therapy produced favorable results. Although tryptophan and histidine produced an early small reticulocyte response in both cases, there was no appreciable increase in the hemoglobin or red cell levels. Thus, even though there are indications that they have some stimulating action on hematopoietic tissue, the results do not justify ascribing the activity of liver to its content of free tryptophane and histidine. Dominici and Penati (27) were also unable to confirm the favorable results of the French authors.

Peabody and Neale (94) stated that histidine and tryptophane are probably not the constituents of liver extract which are effective in eliciting a reticulocyte response in grain-fed pigeons. Since they believed that the pigeon response is a measure of the substance or substances active in the treatment of pernicious anemia, they concluded that liver extracts do not owe their antianemic potency to these amino acids. Inasmuch as the only reliable test for pernicious anemia potency is a clinical one, their conclusion cannot be accepted without reservations.

Tochowicz (110) agreed with Fontès and Thivolle that some of the trouble in pernicious anemia lies in faulty protein metabolism. He found not only impaired absorption of amino acids from the gastro-intestinal tract, in pernicious anemia, but lessened retention of the amino acids by the tissues and their increased elimination in the urine. From the results of blood studies of normal people as well as those suffering from post-hemorrhagic and pernicious anemias, he concluded that although tryptophane may play some role in Biermer's anemia, histidine is of no importance in either the pathogenesis or the treatment of the disease.

Here again there are conflicting reports. Tentatively, one may conclude that tryptophane may play a role in the treatment of pernicious anemia, yet by no means a major one. The evidence for the importance of histidine in the antianemic "factor" is even less convincing.

## XII. MISCELLANEOUS CONTRIBUTIONS

The search for the chemical compound or compounds which are present in therapeutically active liver extract, has, for the most part, centered around the work of the five groups of investigators, so far discussed in some

detail. Less extensive investigations by various other workers should, however, not be ignored. Some of these results have received confirmation, others have not. They will now be briefly discussed.

### 1. *Buchanan on Glutathione*

Buchanan reported: "On January 9, 1929, after many years of speculation and four and one half years of work, I arrived at the point in my investigations where it seemed reasonable to believe that pernicious anemia was due to a yeast" (6). He concluded that the active principle of liver extracts has apparently every chemical resemblance and physiological action of oxidized glutathione. Although Fleming (37) later declared that glutathione, mostly in oxidized form, enters into the composition of the antianemic factor, Koser in 1936 (72) denied the importance of glutathione in this respect.

### 2. *Felix and Frühwein on Liver Fractions*

In 1933, Felix and Frühwein (35) carried out a series of fractionations of liver extract, testing for the presence of activity by determining the reticulocyte response after injection in anemic (not necessarily *pernicious* anemia) or normal persons, and by methemoglobin formation *in vitro*. After extracting hashed liver with  $H_2O$  at pH 4.6–5.0 (acetic acid), they precipitated the protein with tannic acid at pH 3.2. Further fractionation was achieved by treatment with various heavy metal salts, the best results being obtained by precipitating the active principle from  $H_2SO_4$  solution with  $HgSO_4$  in the presence of alcohol and ether. After repeated purification with mercuric sulfate, their preparation still contained sulfur but not phosphorus; the glyoxylic acid reaction for tryptophan and related substances as well as aldehyde reaction proved negative. Biuret reaction was negative; diazo, Sakaguchi and ninhydrin reactions were positive. Total nitrogen calculated on an ash-free basis was found to be 11.85 per cent of which 30.16 per cent was free  $NH_2-N$ . Acid hydrolysis did not increase the  $NH_2$  titer, indicating that the active principle is not a peptide. It is unfortunate that the activity of these preparations was not thoroughly assayed on pernicious anemia patients.

### 3. *Erdős on Liver Fractions*

In 1935, Erdős (32) reported on the preparation of active material from liver and on certain of its properties. Finely hashed liver was first ground with quartz powder and then extracted with dilute  $H_2SO_4$  at different temperatures. The extracts, concentrated at 60–70° C. until the dry weight constituted about 60 per cent, were then mixed with about ten fold volumes of  $H_2O$ , warmed to 50°, cooled, filtered, brought to pH 7.5–8 with  $NaHCO_3$ ,

and again filtered. The opalescent filtrate was acidified with 20 per cent  $\text{H}_2\text{SO}_4$  and concentrated to a dark brown viscous mass. The remaining acid was removed with barium and the excess barium was removed by treatment with  $\text{CO}_2$ . After exact neutralization (pH 7-7.2), the solution was concentrated in vacuo. Finally, after freeing the preparation from protein by use of an iron solution, a powder was obtained, yellow to brown in color, 93.7 per cent soluble in  $\text{H}_2\text{O}$ , 12.1 per cent soluble in warm alcohol, and 0.15 per cent in ether.

*Analysis* (of that portion of the powder which is water-soluble).

	<i>per cent</i>
Total N	4.14
$\text{NH}_2\text{-N}$	0.82
$\text{NH}_2\text{-N}$ after hydrolysis	3.32
Phosphorus	0.65
Sulfur	0.98
Chlorine	0.07
Reduction as glucose	9.0
Ash	0.38
Iron	0.004
Copper	0.0005

It gave no precipitate with sulfosalicylic acid; biuret was positive. The fact that acid hydrolysis increased the  $\text{NH}_2\text{-N}$  by about 300 per cent seemed to indicate a peptide linkage. He believed that his preparation had 6 free amino groups and 18- $\text{CO}\cdot\text{NH}$ -linkages. The titration of the purified preparation supported the supposition of the presence of free carboxyl groups. Erdős was also able to isolate a water-insoluble silver salt from the analysis of which he suggested the formula  $\text{C}_{650}\text{H}_{720}\text{O}_{36}\text{N}_{30}\text{S}_2\text{P}_2\text{Ag}_3$  with a molecular weight of about 10000.

Erdős assayed his material for activity by noting its influence on the anemia produced in dogs and rabbits by the administration of phenylhydrazine (33). In the normal animal, the administration of phenylhydrazine hydrochloride (16 mg. per kg. body weight) lowered the red cell count 25 to 40 per cent in 48 to 72 hours, after which time, spontaneous remission occurred. However, in those animals which received liver preparations along with the phenylhydrazine, the maximal decrease of red cells was reached only after 96 to 120 hours.

In 1942, Erdős modified his previous method of isolation. Finely ground liver was extracted with dilute  $\text{H}_2\text{SO}_4$  at different temperatures and the sulfuric acid was removed with  $\text{Ba}(\text{OH})_2$ . The concentrated filtrate was precipitated with 70% alcohol. The insoluble fraction was removed and the filtrate was concentrated and precipitated with  $\text{AgNO}_3$ . The Ag precipitate was separated and decomposed with  $\text{HCl}$ . The silver free filtrate was concentrated and precipitated with alcohol.



A silver salt was prepared from the alcohol precipitation. 2 grams of dry salt were obtained from 1 kg. of liver.

<i>Analysis.</i>	C	= 67.50
	H	= 6.41
	N	= 14.41
	NH <sub>2</sub> ·N	= 1.4
	S	= 0.99
	P	= 1.06
	Ag	= 5.04

The approximate molecular weight of 6000 was assigned. As for the structure, "it contained three free carbonyl groups, as did the product obtained several years ago. Remaining are six free amino groups, this number increasing to eighteen after hydrolysis." No adequate clinical reports were presented.

#### *4. Jacobs on the Nature of Active Liver Material*

We have thus far discussed the results obtained by investigators who employed the orthodox chemical procedure of isolation and identification. In 1937, Jacobs (54, 55, 56) reported certain conclusions regarding the chemical character of the active liver material which he reached by employing less orthodox means. From a study of the properties exhibited by potent liver extracts, he tried to deduce the chemical nature of the anti-anemic principle and test such deductions by synthesis. Some of the considerations which entered into his reasoning follow:

(1) Since material given by mouth is much less effective than that given parenterally, the active principle may be destroyed in the gut. (This may equally well be due to incomplete absorption of material administered orally.)

(2) Spontaneous remissions may be due to a temporary, partial failure of tryptic digestion.

(3) The function of the active principle may be to modify the course of digestion (particularly tryptic digestion) of normal foods.

(4) Normal, fasting gastric juice reddens an alkaline picric acid solution after 10-15 minutes of heating.

Such a test is negative when applied to the fasting gastric contents of pernicious anemia patients. A positive reaction indicates according to Abderhalden the presence of proteins, proteoses, peptones, or diketopiperazines.

(5) Glucosamine gives a positive picric acid reaction.

(6) The ability of active preparations to be oxidized and reduced suggested to Jacobs the presence of active carbonyl groups.

He cited as evidence the power of preparations to change hemoglobin

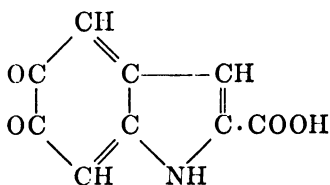
to methemoglobin as reported by Duesberg and Koll (28). However, Deutsch and Wilkinson (26) have found no connection between hematopoietic activity as determined by clinical results and the production of methemoglobin.

(7) The deepening of the reddish color of active preparations on warming indicated to Jacobs a process of polymerization.

The fact that aldehydes form polymers suggested the possibility that such aldehydes were present in active antianemic preparations. In support of his suggestion that the active principle functions by way of modifying tryptic digestion is the evidence that the activity of trypsin is inhibited by aldehyde.

Jacobs reported a reticulocytosis of 14 per cent, 5 days after the subcutaneous injection of an aqueous solution of the product resulting from the interaction of acetaldehyde and glucosamine. Previously, this patient had not responded to ventriculin by mouth. However, since three other previously untreated cases failed to respond to similar treatment, no great importance was attached to the one positive result.

Following the suggestion in his first paper (54) that further investigation of the possible role of glucosamine and the common amino acids (especially leucine and tyrosine) in the pernicious anemia problem might be desirable, he subsequently studied the role of tyrosine (55). Using methods employed by Raper and associates in the identification of substances present in a reaction mixture of tyrosine and tyrosinase, Jacobs concluded that the red substance in liver extract is the 5,6-quinone of dihydroindole-2-carboxylic acid.



Inasmuch as the instability of this compound precluded its synthesis in pure form, impure mixtures were used for clinical test (56). Raper (95) had shown that the 5,6-quinone of dihydroindole-2-carboxylic acid is a product of the action of tyrosinase on tyrosine in the presence of oxygen. Jacobs administered daily such a mixture, prepared from raw potato scrapings and tyrosine, to a pernicious anemia patient. On the fifth day, treatment was interrupted because of the appearance of diarrhea and nausea. The reticulocytes rose from an initial value of 2.8 per cent to 6.0 per cent on the fifth day.

Later Jacobs concluded that the "red substance" was not concerned

in the activity of liver extracts. In his most recent paper, Jacobs (57) proposes to investigate choline for its antianemic effect inasmuch as he was able to isolate this compound from commercial liver extracts.

#### *5. Aleksandrowicz and Gabryelski on Heparin*

Aleksandrowicz and Gabryelski (4) have recently proposed that anti-prothrombin (heparin) is one of the important therapeutic factors in liver preparations. A comparison of some of the properties of the antianemic factor and of heparin showed some similarity in a few respects. According to their hypothesis, pernicious anemia may be caused by the lack of antiprothrombin. One might, therefore, expect to find less of this substance in the blood of a patient than in that of a normal individual. Inasmuch as there is no specific chemical test for heparin, they employed a method based on the fact that, in the presence of heparin, erythrocytes are more resistant to hemolyzing agents. Hence, in the presence of the serum of a pernicious anemia patient, having little or no heparin, the hemolytic action of various substances on the red cells should occur more quickly than in the presence of normal serum; this difference in rate should disappear during successful treatment of the disease. In a study of fifteen pernicious anemia patients and fifteen normal individuals, variable results were obtained, in only two of which the expected trends appeared.

Although it proved inactive in two other cases, intramuscular administration of heparin in one case effected an erythrocyte response, from an initial level of 2.6 millions to 3.9 millions in a four week period. There was no indication of a reticulocyte response at any time during treatment. As Aleksandrowicz and Gabryelski admit, the therapeutic value of anti-prothrombin is not supported by this single case.

They next attempted to test for heparin in liver extracts of proved activity, (those used were called Pernaemon and Sykoton). A positive Molisch reaction, in the absence of glycoprotein, polysaccharides and monoses indicated the probable presence of heparin. Their attempt to isolate heparin in crystalline form from Pernaemon proved unsuccessful.

They cited the following properties exhibited by heparin and an active liver preparation (Pernaemon).

For heparin:

- (1) Immediate precipitation with HCl, HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>—not with strong acetic.
- (2) Immediate precipitation with 5-sulfosalicylic acid (perhaps because this is a moderately strong acid).
- (3) No biuret.
- (4) Precipitated by phosphotungstic acid.
- (5) Positive uric acid test (colors phosphotungstic reagent blue upon warming).

(6) Positive murexide test.

For Pernaemon:

- (1) No precipitate with HCl.
- (2) Precipitate with 5-sulfosalicylic acid.
- (3) Slight biuret, too weak for peptide.
- (4) Turbidity with phosphotungstic acid.
- (5) Positive uric acid test.
- (6) Negative murexide.

Although there is some similarity in certain of the reactions, the lack of complete correspondence, and the non-specificity of the tests, throw doubt on rather than bring support to the hypothesis of Aleksandrowicz and Gabryelski.

*6. Jones, et al. on Nuclear Extractives*

In 1929, Jones, Phillips, Larsell, and Nokes (65) reported the hematopoietic effect of nuclear extractives in human anemias. They believed that nuclear extractives from various organ sources—considered to be nucleoproteins and the sodium salts of nucleic acids—contained an unknown hematopoietic stimulant. Oral administration of such extractives, prepared from chicken corpuscles, beef spleen, beef liver, beef kidney, beef heart muscle, salmon liver, beef thymus and beef pancreas, in  $\frac{1}{4}$  g. doses, yielded satisfactory results in pernicious as well as other anemias. They thus tentatively concluded that the hematopoietic stimulant is an integral part of the cell nucleus.

### XIII. CRITERIA OF ACTIVITY

In the foregoing sections, the methods of preparation and, in some cases, the properties of various antianemic preparations have been described. Unfortunately, for the reader and the authors, agreement does not as yet exist among the different investigators regarding what is the active material, or what are its properties. It may be of some interest, however, to compare the relative activities of some of the better preparations. How should such a comparison be made? As we have already stated, the phenomenon of reticulocyte response is of little value in determining the comparative efficiency of extracts. It may or may not give an indication of the presence of potency, and it does not appear to indicate the degree of potency. Murphy suggests (92) that perhaps the most critical and important means of comparison is the determination of the amount of antianemic material that is necessary to maintain the erythrocyte count of a pernicious anemia patient at a normal level. Such observations should be made for at least six months, or longer. In such comparisons, we must also recognize the fact that it takes an appreciably greater amount of material to maintain the levels at five millions or higher than it does to maintain them at

lower levels. Inasmuch as there are few reports available concerning the long-time maintenance requirements of the extracts in which we are interested, we cannot make our comparisons on this ideal basis.

Schales attempted to develop a method of evaluating the activity of commercial liver extracts independent of clinical test (97, 98). Among the properties which he found of no or little use as indicators of potency might be mentioned: the dry weight, free hexoses of glucose type, N content of the tested extracts, and freezing point lowerings. The specification of from how much fresh liver the preparation comes cannot be used here, inasmuch as the amount of the antianemic principle in fresh liver depends somewhat on the season, feeding and age of the animal, as well as other variable factors. Even if the same liver is used as the starting point, the concentration of the active material in the final preparation varies with the different methods of purification.

He found that liver extracts can be approximately standardized by the weight and nitrogen content of the material soluble in 70 per cent alcohol and precipitated by 90 per cent alcohol (Cohn's fraction G). He based his method on the fact that parenteral administration of 80 mg. purified Dakin and West material (Anahaemin) elicited a maximal reticulocyte reaction. From the organic residue and nitrogen content of fraction B (Schales), he evaluated the liver extracts in terms of the preparation of Dakin and West (1935) which had a nitrogen content of 15.3 per cent. Thus, the maximal content of active material (considering the Dakin-West product as pure antianemic substance) in a tested extract would be given by the formula:

Maximal Content (D and W Substance)

$$= \frac{\text{Organic residue in mg. of fraction B} \times \text{percentage N content}}{15.3}$$

Since 80 mg. D + W Substance yielded a maximal reticulocytosis, he further calculated how much of the tested extract must be *minimally* injected to get the same clinical response.

$$\text{Minimal Amt. (cc.)} = \frac{80}{\text{Maximal content (D + W Substance)}}$$

where the D + W Substance is expressed in terms of mg. per cc.

However, a difficulty with this method of comparison is that fraction B has impurities with varying nitrogen contents, the magnitudes of which differ in the different preparations. Since Anahaemin has been shown to be impure (23), it can hardly be accepted as a standard with which all other preparations are compared.

## XIV. COMPARISON OF ACTIVITIES OF PREPARATIONS

In the absence of a more satisfactory basis of comparison, a few of the preparations may be compared in terms of erythrocyte response elicited by definite quantities of different products (see Fig. 3). Inasmuch as the size of response varies with the initial red blood cell level, an attempt has been made to compare cases in which the initial erythrocyte levels were about 1.2 to 1.8 million. Responses reported by Murphy (92) and Hartfall

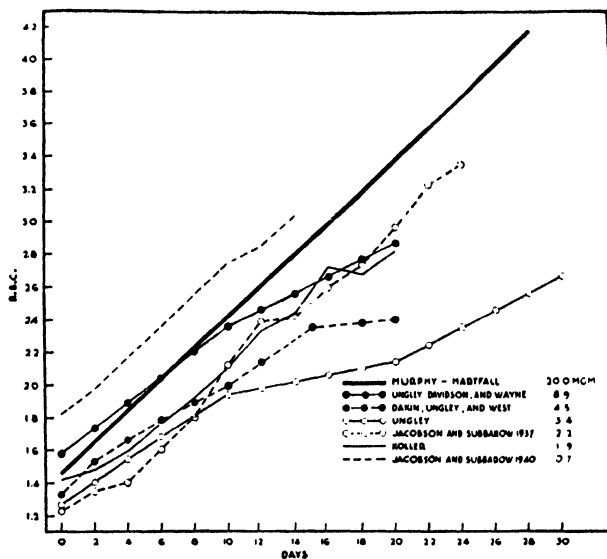


Fig. 3

## Average Erythrocyte Regeneration Curves Following the Administration of Various Purified Liver Extracts

The calculated average daily amount of material administered is recorded in the legend. The sources of these data are contained in the text. (The daily doses recorded for the curves of Jacobson and Subbarow refer only to the amount of primary factor administered; in addition, three accessory factors were administered in the 1937 curve, in a daily amount of 3.4 mg. and five accessory factors were administered in the 1940 curve in a daily amount of 6.2 mg.)

(49) following the use of highly active extract have been used as a basis of comparison. For the individual preparations of the several authors, each curve represents the average of the responses of a number of patients.

From Figure 3 it is to be noted that the curves of Ungley, Davidson and Wayne and of Dakin, Ungley and West based on the administration of daily doses of 8.9 and 4.5 mg. respectively, differ significantly from one another and even the larger dose does not insure the maximal response.



Koller's curve, based on a dose of only 1.9 mg. a day, indicates good activity but the more purified preparations such as his fractions numbered 25, 28, 35 do not show the same activity.

The data have been presented in the hope that further advances in the understanding of the chemical nature of the substances concerned will enable reconciliation of the discrepancies in the magnitudes of response achieved by different investigators. It is, unfortunately, apparently not possible at the present time to reconcile the various claims and facts regarding the material or materials which are present or capable of extraction from liver, and which are therapeutically active in pernicious anemia.

### XV. GENERAL SUMMARY

The reader has probably been impressed with the great variation in the chemical properties of the preparations to which activity has been attributed. Some of these have been summarized in Table XX.

The reasonable explanation for these differences is that the various preparations are still more or less contaminated and the activity of the different products may be due to one unknown substance which is present in all these products in small amounts. However, the chemical properties of these various materials vary with the nature of the impurities in the various preparations.

Although it is not yet possible to present the properties of the anti-pernicious anemia material from liver with chemical exactness, it is, nevertheless, proper to note that during the seventeen years since whole liver therapy has been introduced, the amount of material needed by the patient per day has decreased from 400 g. to less than 1 mg. Such progress makes it reasonable to expect the isolation and identification of the active material to be an attainable objective.

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# The Mechanism of Action and Metabolism of Gonadotropic Hormones in the Organism<sup>1</sup>

By BERNHARD ZONDEK AND FELIX SULMAN<sup>2</sup>

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## I. INTRODUCTION

A study of the mechanism of action and the metabolism of gonadotropic hormones is of especial interest because they are primary hormones which initiate a hormonal chain reaction in the body. It is possible that an elucidation of their action will allow closer insight into the mechanism of other tropins of the pituitary gland, thus furnishing valuable information. Furthermore, the test methods for the action of the gonadotropic hormones and their metabolites have been so well developed that information of a precise nature may be obtained through their use.

Whereas the biological effect of some hormones, *e.g.*, adrenalin, insulin, pitocin, pitressin, and intermedin, becomes apparent very shortly after their administration, sex hormones act differently. There is a marked time interval between the administration of the sex hormones and their first perceptible effect. The duration of this period cannot be shortened either by changing the route of administration or the solvent or by increased dosages.

<sup>1</sup> Due to wartime restrictions only selected subjects could be reviewed here.

<sup>2</sup> Part of the work under review was carried out with the aid of the Rockefeller Foundation and the Ella Sachs Plotz Foundation, to whom our thanks are due.

What happens in the interval between the administration of the hormone and the time when it takes effect in the organism? Is it stored in the organism or transformed to an intermediate substance or metabolite or is it rendered inactive or destroyed immediately? The following sections deal with the data available on this problem.<sup>\*</sup>

## II. TIME INTERVAL REQUIRED FOR INACTIVATION OF GONADOTROPIC HORMONES

It is well known that prolan, the gonadotropic hormone from pregnancy urine (*chorionic gonadotropin*) rapidly disappears from the blood after its injection.

Ehrhardt (1930) injected up to 700 cc. of pregnancy blood intravenously into men or non-pregnant women. Two cc. of blood from the infused patients, 2-16 hours later, contained 1 MU chorionic gonadotropin L.H. and up to 24 hours later 1 MU chorionic gonadotropin F.S.H. would be found. Calculation of these values gives a 30% recovery of pregnancy blood gonadotropin in man after 24 hours. Lipschuetz and Vivaldi (1934), Vivaldi (1934), and Lipschuetz, Fuente-Alba, and Vivaldi (1935a) investigated the disappearance of chorionic gonadotropin from the blood of rabbits which received 100 rabbit units intravenously. They calculated that 80% of the dose had disappeared within 6-8 hours and 95% after 10 hours. The curve of elimination of chorionic gonadotropin constructed by them took a logarithmic course. Stamler (1937) found that 3 hours after the intravenous injection of chorionic gonadotropin into a dog 38.4% remained in the blood. A gelding injected with 300,000 U intravenously, showed 3000 U per liter blood up to 2 hours later, 1000 U up to 8 hours later, and 800 U up to 22 hours later.

Other approaches of a precise nature to the problem of inactivation of chorionic gonadotropin in the organism have been made in two ways:

- (I) Injection of chorionic gonadotropin into animals and recovery of the hormone from their carcasses by extraction after various time intervals;
- (II) A certain amount of chorionic gonadotropin was injected into immature female rats after which smaller amounts of an antiserum to the gonadotropin were given at various intervals. The minimal dose of antigonadotropin required to prevent the gonadotropic reaction at any given interval indicates the amount of chorionic gonadotropin circulating in the body at that time.

Both methods have been described by Zondek (1940), Zondek, Sulman, Sklow (1940/41), and Zondek and Sulman (1942).

A summary of the results obtained with methods I and II as tabulated in Table I demonstrates the rate of chorionic gonadotropin breakdown

<sup>\*</sup> A short review of this topic was given by the senior author in 1941.

**Amount of Chorionic Gonadotropin (CG) Circulating in the Body of the Rat at Different Intervals after Injection**

Method I		Method II						
Assay rat	CG recovered by extraction	Assay rat	CG injected first	Anti-CG subsequently injected	Interval between injections	Gonadotropic reaction		
no.		no.	(IU)	(anti-units)	(hours)	I Vaginal estrus	II Blood dots	III Corpora lutea
1	100% after 0 hour	1	5	5	0	—	—	—
		2	5	3.75	0	+	—	—
		3	5	2.50	0	+	—	+
		4	5	1.25	0	+	—	+
		5	5	0.50	0	+	+	+
2	50% after 1 hour	6	5	5	1	—	—	—
		7	5	3.75	1	—	—	—
		8	5	2.50	1	±	—	—
		9	5	1.25	1	+	—	—
		10	5	0.50	1	+	—	+
3	30% after 4 hours	11	5	5	4	—	—	—
		12	5	3.75	4	—	—	—
		13	5	2.50	4	—	—	—
		14	5	1.25	4	+	—	—
		15	5	0.50	4	+	—	—
4	25% after 8 hours	16	5	5	8	—	—	—
		17	5	3.75	8	—	—	—
		18	5	2.50	8	—	—	—
		19	5	1.25	8	±	—	—
		20	5	0.50	8	+	—	—
5	20% after 12 hours	21	5	5	12	—	—	—
		22	5	3.75	12	—	—	—
		23	5	2.50	12	—	—	—
		24	5	1.25	12	—	—	—
		25	5	0.50	12	+	—	—
6	15% after 18 hours	26	5	5	18	—	—	—
		27	5	3.75	18	—	—	—
		28	5	2.50	18	—	—	—
		29	5	1.25	18	—	—	—
		30	5	0.50	18	+	—	—
7	10% after 24 hours	31	5	5	24	±	—	—
		32	5	3.75	24	±	—	—
		33	5	2.50	24	±	—	—
		34	5	1.25	24	±	—	—
		35	5	0.50	24	±	—	—
8	5% after 30 hours	36	5	5	30	+	—	+
		37	5	3.75	30	+	—	+
		38	5	2.50	30	+	—	+
		39	5	1.25	30	+	—	+
		40	5	0.50	30	+	+	+

within the body of the rat. The results obtained for the gonadotropic reaction I (vaginal estrus) were generally the most reliable. Rat 1 (method I) shows that 100% of the gonadotropin injected into the assay rat could be recovered by our extraction method. Rats 1-5 (method II) were included to check the titer: they show that if gonadotropin and antigonadotropin are given simultaneously, 5 IU of the former are neutralized by 5 anti-units<sup>4</sup> of the latter. Rat 8 (method II) shows that a maximum of 2.5 IU were present in the body 1 hour after the injection of 5 IU, which corresponds to the 50% recovery in method I. Rat 14 of method II demonstrates that after 4 hours, more than 1.25 anti-units are required to inactivate the previously injected 4 IU of gonadotropin; this is in accordance with the 30% recovery in method I. Similarly 8, 12, 15 hours after the injection

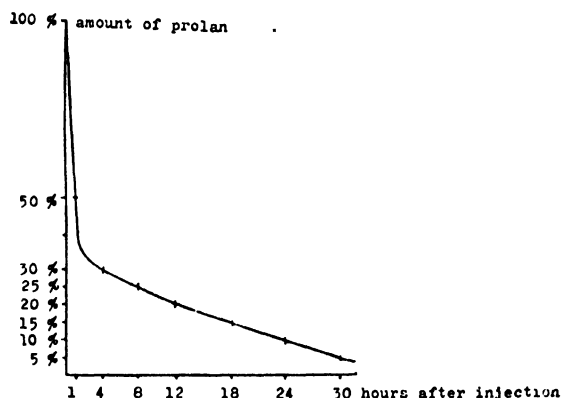


Fig. 1. Curve of the Inactivation of Chorionic Gonadotropin in the Body of the Rat  
(From Zondek, 1941)

of 5 IU of gonadotropin, more than 1.25, 0.5, and 0.5 anti-units, respectively, had to be given to inhibit the gonadotropic reaction; these figures also agree with the 25%, 20%, and 15% recoveries found with method I.

If 24 hours were allowed to elapse after the chorionic gonadotropin injection the injection of antiserum was found to be ineffective (Rats 31-35), owing to the fact that the gonadotropic reaction of the ovaries had already started and the estrogen produced by the matured follicles had entered the blood, producing estrus. Even the injection of a ten-fold quantity of anti-units at this time did not inhibit the  $\pm$  vaginal reaction. Thirty hours after the gonadotropin injection it was impossible to check the gonadotropic reaction and hence method II was no longer applicable. Method I shows that after 24 hours only 10% and after 30 hours only 5% of the injected hormone was still present in the body.

<sup>4</sup> An AU (anti-unit) is the amount of antigonadotropin necessary to neutralize the effect of 1 IU of chorionic gonadotropin in the immature female rat.

The initial rapid elimination and subsequent gradual decrease in the amount of hormone present in the body are graphically shown in the logarithmic curve, Fig. 1, derived from the results obtained by methods I and II.

Whereas this curve is based on the rate of gonadotropin elimination when small quantities (5 IU) are injected (method II), it should be stressed that the same curve may be constructed for the rate of destruction of chorionic gonadotropin when large quantities are employed (250 IU, method I). It seems that a general rule of inactivation prevails. This may be stated as follows: An excessive amount of hormone administered to the body is reduced within one hour to half the original amount. When the irreversible reaction of the stimulated organ starts within 24-27 hours, only a small part (10%) of the hormone still remains in the body. The rest has meanwhile been eliminated without appearing in the urine. This phenomenon may be called: "the initial drop during the latent period," and may be defined as the rapid elimination of excess hormone administered to the body, to a minimum level, during the time-lag before the gonadotropic reaction itself has set in. This rule also seems to hold for other sex hormones such as estrone, stilbestrol, and progesterone (Zondek, 1934a, b, 1939; Zondek and Sulman, 1939).

*Pregnant mare blood gonadotropin*, when injected, behaves quite differently, probably because of its inability to pass the renal epithelium in sufficient amounts. Catchpole, Cole, and Pearson (1935) injected rabbits intravenously with 30 cc. of pregnant mare blood gonadotropin containing about 3,000 IU gonadotropin. A reduction of only 50% of the titer was found as late as 24-26 hours after injection. A castrated horse which received 3 l. containing about 90,000 IU of pregnant mare blood gonadotropin showed only a 50% reduction of the hormone titer as late as 6 days after injection. According to Cole and Saunders (1935) 50% reduction may appear in mares as late as 8-10 days after injection. The delayed excretion of pregnant mare blood gonadotropin from the body furnishes an explanation for the finding of Cole, Guilbert, and Goss (1932) that single doses of this hormone are as effective in their action on the gonads as split doses.

The injection of *pituitary gonadotropin* has been studied by Evans, Simpson, and Austin (1933) who found a somewhat delayed excretion in rhesus monkeys. Seidlin (1940) injected guinea pigs with dog pituitary gonadotropin; his experiments will be discussed later. A detailed report on the blood clearance of horse pituitary gonadotropin injected intravenously into rabbits has been given by Robson and MacPherson (1940). The average amount recovered in the blood was 40% after 6 hours, 28% after 12 hours and 9% after 24 hours. In pregnant rabbits the results were uncertain.



In a child suffering from meningocele the amount recovered in the blood after 24 hours was only 6%.

The rapid destruction of gonadotropins in the organism may be slowed down by Parkes' method of *implantation* of hormone pellets. Parkes (1942) found that the gonadotropic effect persisted for a maximum duration of 6 days when pellets containing 200 IU of chorionic gonadotropin in 90% cholesterol were implanted into animals (see also Parkes and Emmens, 1944).

Another way of prolonging the resorption of gonadotropins has been studied by Sulman, Levy-Hochman, and Black (1945) who used insoluble gonadotropin tannate made up into pellets. The average duration of effectivity of pellets containing 100 IU of various gonadotropins implanted intramuscularly into infantile female rats is shown in Table II. Details will be given later.

TABLE II  
*Effectiveness of Gonadotropin Tannate Pellets Implanted into Rats*

Gonadotropin pellet	Chorionic gonadotropin	Pregnant mare blood gonadotropin	Ox pituitary gonadotropin
	<i>days</i>	<i>days</i>	<i>days</i>
Native after alcohol precipitation.....	1	1	2
Tannate with 50% cholesterol.....	5	Not studied	4
Tannate alone.....	7.5	6	6

### *Summary*

The rate of inactivation of *chorionic gonadotropin* injected into the body is expressed by a logarithmic curve. The most striking feature of this curve is the initial fall in the amount of the chorionic gonadotropin injected, which amounts to a decrease of 50% within the first hour. Later on, the curve declines more gradually to reach a 10% level 24 hours following the subcutaneous injection. At this time the ovary commences to undergo irreversible gonadotropic changes. The initial rapid decrease of the chorionic gonadotropin in the body during the time-lag may be termed the 'initial drop during the latent period.' The amounts of gonadotropin recovered by different authors are compiled in Table III. They differ with the different species studied and the hormone used. Hormones originating from the *blood* either of pregnant mares or women are apparently more slowly eliminated than chorionic gonadotropin from urine, especially if injected in the donor species. The rate of clearance of (horse) *pituitary gonadotropin* from the blood lies between that of chorionic gonadotropin which is cleared more rapidly and of pregnant mare blood gonadotropin, which disappears from the blood much more slowly.

TABLE III

*Recovery in Blood of Injected Gonadotropins (Various Authors)*

Author	Gonadotropin	Species injected	Hormone recovery	
			hours	per cent
Ehrhardt (1930)	Chorionic gonadotropin (blood)	Man	24	30
Lipschuetz, Vivaldi (1934); Lipschuetz, Fuente-Alba, Vivaldi (1935a)	Chorionic gonadotropin (urine)	Rabbit	6-8	20
			10	5
Stamler (1937)	Chorionic gonadotropin (urine)	Dog	3	38.4
		Gelding	2	50
			8	16.5
			22	13.5
Zondek, Sulman, Sklow (1940/41)	Chorionic gonadotropin (urine)	Rat	1	50
			4	30
			8	25
			12	20
			18	15
			24	10
			30	5
Catchpole, Cole, Pearson (1935)	Pregnant mare blood gonadotropin	Rabbit	24	50
			48	25
			72	12.5
			96	6.25
			120	3.125
			144	1.5
			168	0.75
		Gelding	144	50
			216	25
Cole, Saunders (1935)	Pregnant mare blood gonadotropin	Mare	240	50
Robson, MacPherson (1940)	Horse pituitary gonadotropin	Rabbit	6	40
			12	28
			24	9
		Map	24	6

The rapid destruction—especially of chorionic gonadotropin—may be slowed down to 6 days by using cholesterol-gonadotropin pellets and to 7.5 days with gonadotropin tannate pellets.

## III. EXCRETION OF GONADOTROPIC HORMONES

Ehrhardt (1930) who injected *chorionic gonadotropin* (700 cc. pregnant women's blood) into non-pregnant patients was the first to describe its excretion by the kidney. The patients' urines gave positive Aschheim-Zondek Tests during the first 24 hours after infusion; during the next 24 hours, only FSH was excreted in the urine. Chorionic gonadotropin excretion ceased somewhat later. The hormone excretion began as early as 10 minutes after the infusion (*cf.* Ehrhardt and Ruhl, 1933).

Snyder and Wislocki (1931) who injected rabbits with chorionic gonadotropin mentioned its excretion in the urine. Hill and Parkes (1931) injected pregnant rabbits intravenously with chorionic gonadotropin. The placentae of these animals (normals and hypophysectomized) were found to have a rather high gonadotropin content. These experiments demonstrate a special ability of the placenta to fix gonadotropin or to enhance its effect, since it should be borne in mind that rabbit placentae and the blood of pregnant rabbits do not contain any gonadotropin factor. These results were supplemented by Goodman and Wislocki (1933) who administered chorionic gonadotropin intravenously to pregnant rabbits and cats. Subsequently, they could not detect any gonadotropic hormone in either the amniotic or the allantoic fluids.

Sklow (1942), in our laboratory, studying the permeability of the human placenta to the (chorionic) gonadotropin formed therein compared the concentration of hormone in the umbilical vein (fetal) and the retro-placental blood (maternal). He found a much higher concentration of gonadotropin in the maternal blood (17,000 RU = 4,000 IU on an average) as against 745 RU = 160 IU in the umbilical vein. The proportion is thus about 25:1. These results, together with those mentioned previously, suggest the possibility that the placenta is able to adsorb and store chorionic gonadotropin.

Evans, Simpson, and Austin (1933) demonstrated the excretion of injected chorionic gonadotropin in the urine of rats and rhesus monkeys. Parkes and White (1933) injected chorionic gonadotropin intravenously into rabbits and found that about 33% of the dose was excreted in the urine within 9 hours. They state that female rabbits can excrete about 10 rabbit units of chorionic gonadotropin per kg. per 24 hours. This rate of excretion would be of the same order (per kg. body weight) as the output of pregnant women. The objection must be raised, however, that these quantities considerably exceed those found by all other authors. Quantitative investigations by Zondek (1935) have shown that up to 5% of subcutaneously injected chorionic gonadotropin may be excreted in the urine of the rabbit or rat, and in human beings excretion may reach 10%. Stamler (1937) found in dogs that the hormone appeared in the urine within 1

minute after its administration, that its excretion continued for over 20 hours, and that the total amount excreted in the urine was 11.2%. In the gelding, Stamler found more than 5% of the administered chorionic gonadotropin in the urine. Friedman and Weinstein (1937) reported that no more than 20% of a dose of chorionic gonadotropin, repeatedly injected intramuscularly into men, could be recovered in the urine. After large doses by mouth (8,000–40,000 U) none was detected in the urine.

Zondek, Sulman, and Sklow (1940/41) studied the urine excretion of chorionic gonadotropin in rats by a special technique. Five female rats had their urinary bladders ligated between the *collum vesicae* and the vagina. Immediately afterwards 1000 IU of chorionic gonadotropin were given subcutaneously in 0.5 ml. normal saline. At intervals of 1, 2, 4, 20 and 24 hours the rats were sacrificed and the urine was collected from their bladders and assayed for chorionic gonadotropin. After 4 hours they found 1% and after 20 hours 2% of the amount administered. In a second assay on 5 male rats, the rats had the urethra ligated subcutaneously and immediately afterwards they were injected with the same amount of gonadotropin dissolved in 2 ml. of distilled water in order to cause strong diuresis. At intervals of 1, 2, 4, 20 and 24 hours the rats were killed, their bladders emptied and the chorionic gonadotropin content in the urine was determined. After 20 hours 5% of the injected gonadotropin was recovered. Before and after that interval none was found. A similar technique was used by Robson and MacPherson (1940) in rabbits without, however, obtaining conclusive results because of intoxication of the test animal.

Salmon and Hamblen (1943) studied the recovery in the urine of chorionic gonadotropin injected intravenously into rabbits. Excretion was terminated after 96 hours, the total amount excreted reaching 5.7%. The bulk was excreted within 24 hours.

Sulman, Levy-Hochman, and Black (1945) described a way of *prolonging* the *resorption* and urinary excretion of chorionic gonadotropin by the use of chorionic gonadotropin tannate. Control rabbits receiving native chorionic gonadotropin in aqueous solution intramuscularly, excreted 5% of it within 1 day. Rabbits, injected with chorionic gonadotropin tannate intramuscularly, excreted 5% of it on the 5th day after injection. Rabbits implanted with pellets of chorionic gonadotropin tannate excreted 5% as late as the 6th day after implantation. On all the other days only subthreshold doses of chorionic gonadotropin appeared in the urine, indicating that the whole amount excreted did not much exceed the 5% found on those days when the bulk was excreted. These experiments show that combination with tannate delays chorionic gonadotropin absorption and excretion considerably.

As to *pregnant mare blood gonadotropin* it has already been mentioned that after its injection into rabbits or geldings it disappears very slowly from the blood and is not excreted in the urine or feces (Catchpole, Cole, and Pearson, 1935). Evans, Simpson, and Austin (1933) who injected pregnant mare blood gonadotropin into rats and rhesus monkeys detected only traces of the hormone in the urines of these animals. This hormone does not pass through the glomerular epithelium probably owing to the rather large size of its molecule. This agrees with the fact that in the pregnant mare large amounts of this hormone are present in the blood, while only very little can be detected in the urine.

Experiments concerning the urinary excretion of injected *pituitary gonadotropins* have been reported by Evans, Simpson, and Austin (1933) in rhesus monkeys. Seidlin (1940) injected guinea pigs with hog pituitary gonadotropin and recovered part of it in the urine of the animals, especially in castrates (*cf.* below). Robson and MacPherson (1940) administered horse pituitary gonadotropin intravenously to rabbits and found a rather rapid disappearance from the blood, yet urinary excretion accounted for only part of the hormone administered. After 6 hours they recovered 10–16%, after 12 hours about 18.5%, and after 24 hours 14% from the urine. In a child suffering from meningocele the recovery from the urine after 24 hours amounted to 4%.

### Summary

The urinary excretion of injected chorionic gonadotropin ranges, on an average, between 5–10%. In rare cases more has been observed. The values found by different authors are given in Table IV.

A considerable delay of resorption and urinary excretion of chorionic gonadotropin has been obtained with chorionic gonadotropin tannate. The bulk of excretion (5%) was observed on the following days after administration:

*native* gonadotropin injected intramuscularly in solution: 1st day,  
gonadotropin *tannate* injected intramuscularly in suspension: 5th day,  
gonadotropin *tannate* implanted intramuscularly as *pellets*: 6th day.

Chorionic gonadotropin injected into pregnant rabbits is selectively stored in their placentae, but is not excreted into the urine or the amniotic or allantoic fluids. Human placenta stores and adsorbs chorionic gonadotropin.

Pregnant mare blood gonadotropin appears in the urine only in traces because of its molecular size.

Pituitary gonadotropin seems to be excreted in the urine more than other gonadotropins, but the data available on this subject are scarce.

## IV. SITE OF INACTIVATION OF GONADOTROPIC HORMONES

It is well known that steroid hormones such as the estrogens and androgens are inactivated by the liver and the spleen (Zondek, 1934, 1935, 1941, and others) but little information is available about the site of inactivation of the gonadotropins. This question is of importance for an understanding

TABLE IV  
*Urine Excretion of Gonadotropin in Various Animals*

Author	Gonadotropin	Species injected	Hormone recovery in urine	
			hours	per cent
Parkes, White (1933)	Chorionic gonadotropin	Rabbit	9	33
Zondek (1935)	Chorionic gonadotropin	Rabbit	24	5
		Rat	24	5
		Man	24	10
Stamler (1937)	Chorionic gonadotropin	Dog	20	11.2
		Gelding	24	5
Friedman, Weinstein (1937)	Chorionic gonadotropin	Man	24	20
Zondek, Sulman, Sklow (1940/41)	Chorionic gonadotropin	Rat	4	1
		Rat	20	2-5
Salmon, Hamblen (1943)	Chorionic gonadotropin	Rabbit	24	3.5
			48	1.7
			96	5.7
Catchpole, Cole, Pearson (1935)	Pregnant mare blood gonadotropin	Rabbit	0	0
		Gelding		
Robson, MacPherson (1940)	Horse pituitary gonadotropin	Rabbit	6	13
		Rabbit	12	18.5
		Rabbit	24	14
		Man	24	4

of the action of the pituitary hormones in general. The question arises whether pituitary tropins act like catalysts or enzymes, in which case they would not necessarily be used up during their activity; or whether their action is similar to that of, *e.g.*, acetyl choline which is destroyed by its esterase immediately after exerting its effect. The latter view receives

support from the observation that only about 10% of the gonadotropin injected can be recovered from the blood and urine.

Lipschuetz, Fuente-Alba, and Vivaldi (1935b), by means of nephrectomy, studied the importance of urine excretion in the inactivation of *chorionic gonadotropin*. Whereas in normal rabbits 80% of the chorionic gonadotropin disappeared from the blood within 10 hours of intravenous injection, only 30% was missing in nephrectomized rabbits. This would imply that the difference, *i.e.* 50%, is either excreted or inactivated by the kidney. On the other hand, the intoxication occurring in nephrectomized animals may represent a secondary factor which might depress the inactivating power of another organ in the body. Hence experiments in nephrectomized animals are difficult to evaluate. We have studied the inactivation of chorionic gonadotropin in nephrectomized rats as early as 4 hours after nephrectomy at a time when gross symptoms of intoxication were not yet noticeable. Whereas 30% of the hormones was recovered from the blood in the controls, 80% was recovered in the nephrectomized rats (Zondek and Sulman, 1944).

Chorionic gonadotropin is not inactivated *in vitro* by liver (Zondek, 1935), or by spleen or muscle (Zondek, 1940), or by kidney, testes, and ovaries (Zondek and Sulman, 1944). Twenty g. portions of a macerate of these organs were mixed with 1000 IU chorionic gonadotropin dissolved in 20 cc. of phosphate buffer (pH 7.9). The mash was then allowed to stand in the incubator for one hour. Subsequently, it was shaken for an hour and again placed in the incubator for the same period of time. Extraction of chorionic gonadotropin is done in the following way: an acetone dry-powder is prepared from the tissue mash, this is extracted with 50 cc. *N*/20 NaOH and 50 cc. *N*/20 HCl for three hours each and subsequently extracted with 60 cc. distilled water. All extracts are then mixed and the resulting fluid tested for its gonadotropic activity on infantile mice or rats. In the control experiments as well as in those incubated, 100% of the chorionic gonadotropin administered could be recovered. Similar attempts at inactivation were carried out with organ slices shaken for 5 hours at 37°C. All of them proved that chorionic gonadotropin is not inactivated *in vitro* by liver, muscle, spleen, kidney, testes, and ovaries.

Selye (1940) studied the question whether the ovary, as the target organ of the gonadotropic hormones, destroys the hormones while they exert their action upon it. He cites the observation of A. E. Adams (1930), that removal of one ovary which causes compensatory hypertrophy of the remaining gonad has been looked upon as an expression of the effect of the total amount of anterior pituitary hormone being available for one ovary. This hypertrophy might, however, also be looked upon as resulting from increased gonadotropic hormone secretion by the hypophysis of the

partially spayed animal. He, therefore, reinvestigated the compensatory hypertrophy in hypophysectomized and unilaterally ovariectomized rats. It was found that combined injection of chorionic gonadotropin and pregnant mare blood gonadotropin in these animals caused the same increase in weight of the single ovary as the same quantity of hormone did in each ovary of hypophysectomized rats possessing both their gonads. Hence, he concluded that no significant destruction of the gonadotropic hormones takes place in the ovary.

Since Seidlin (1940) and Glaser and Cohen (1940) have demonstrated that gonadotropic and thyrotropic hormones of the hypophysis are inactivated by their respective target gland tissues, it seemed worth studying the rate of chorionic gonadotropin inactivation in the body of animals with the target organ removed. These experiments were recently carried out with the following results (Zondek and Sulman, 1944). Ovariectomized adult rats inactivated 1000 IU chorionic gonadotropin, injected subcutaneously, as quickly as the controls. The same results were obtained in adrenalectomized rats as well as in spayed and adrenalectomized rats.

Another question is whether the *reticulo-endothelial system* (RES) inactivates the gonadotropins. In answer to this problem we may refer to the investigations of Gordon and his collaborators (Gordon, Kleinberg, and Charipper, 1937, 1939; Gordon, 1937) who found that the formation of antibodies to the gonadotropic hormones is effected in the reticulo-endothelial system. It would seem possible that the reticulo-endothelial system is also able to inactivate the gonadotropins. Emery (1937) investigated the effect of splenectomy in rats on the reaction provoked by pituitary gonadotropin grafts. He did not find any increase or decrease of the reaction in the splenectomized animals.

These results as to the function of the spleen in the metabolism of gonadotropins are in concurrence with those of Zondek and Sulman (1944) who worked with *chorionic gonadotropin*. They compared the minimum quantity of chorionic gonadotropin which was needed to evoke follicle maturation, vaginal estrus, and corpus luteum formation in normal immature female rats and in splenectomized immature female rats. No difference was found between the two series of assays. The failure of spleen to inactivate chorionic gonadotropin *in vitro* has been reported above. A further study of the influence of the spleen on the rat's own *pituitary gonadotropin*, by splenectomy of immature female rats, showed that the splenectomized animals, as compared with the controls, did not show any acceleration of the onset of maturity. Nor was this effect obtained when injections of progonadotropic serum were added. The latter serum had been obtained from a rabbit protractedly injected with rat pituitary extract. As these 3 methods of assay yielded negative results, another



method was studied by the authors. It was found that splenectomized rats and rabbits did inactivate chorionic gonadotropin much more slowly than normal rats. The rabbits were injected intravenously with 1000 IU of chorionic gonadotropin, while the rats received the same amount intramuscularly. The hormone recovery from the blood was studied 3-4 hours following injection with the result that double the amount was found in the splenectomized animals. Similar results were obtained with rats previously treated with trypan Blue. When splenectomy was combined with blockage of the RES by trypan Blue 4 times as much hormone could be recovered from the blood of the animals thus treated. Controls were always run with normal animals of the same size and sex. These experiments prove that there is a mechanism for the inactivation of injected gonadotropin which is governed by the RES and the spleen. This mechanism works only *in vivo* and not *in vitro*.

There are earlier observations indicating that there might be a connection between the spleen and the metabolism of the gonadotropic hormones, but they are full of contradictions and partly unfounded. The spleen was found to increase in weight and size during pregnancy, and before menstruation and estrus (Anufrejew, 1910); it increases also after castration (Masui and Tamura, 1925). All these four conditions may admittedly be connected with a higher level of chorionic gonadotropin in the body. That splenectomy provokes earlier maturity has been maintained by Aschner (1918), Radosavljevitch and Kostitch (1929), Matteace (1936) and Ssacharow (1936), but has been refuted by Kehrer (1937). The injection of spleen extracts is said to delay sexual maturity (Radosavljevitch, Kostitch, and Vlatkovitch, 1931, Matteace, 1936). Sauerbruch and Knake (1937) found an increased excretion of chorionic gonadotropin in the urine of splenectomized men and animals. They assume also a higher blood level of chorionic gonadotropin after splenectomy, without, however, giving detailed evidence.

Catchpole, Cole, and Pearson (1935) found that the rate of disappearance of *pregnant mare blood gonadotropin* from the blood of rabbits and horses injected with this preparation was not changed by castration. Extraction of the uteri, spleens, lungs, kidneys, and livers of the rabbits showed that there was no appreciable storage of the hormone in any of these organs. Seidlin (1940) studied the effect of 30 minutes' contact at room temperature (23°C.) of macerated hog ovary and liver with pregnant mare blood gonadotropin. It seemed that both tissues had only a slight inactivating effect, if any, on the gonadotropin.

McPhail, Parkes, and White (1933) studied the disappearance of *pituitary gonadotropin* in two female rabbits, whose blood circulations had

been joined by a cross-circulation device. If one rabbit was mated the gonadotropic stimulus released from the mated rabbit's pituitary stimulated only the ovaries of the mated animal, not those of the cross-circulated one. If the mated rabbit was spayed immediately after copulation the cross-circulated animal showed ovulation. The authors conclude:

"It would appear that the ovaries begin to withdraw the hormone from the circulation long before the full amount necessary to cause ovulation has been secreted and that ovaries left in the mated partner withdraw an appreciable amount before cross-circulation can be established."

Similar results were already reported in 1932 by Brambell and Parkes without comment. It should be remembered, however, that Selye (1940) found that this mechanism does not hold for chorionic gonadotropin and pregnant mare blood gonadotropin in rats.

The experiments of the London group have, however, found some corroboration in the work of Seidlin (1940) who incubated hog pituitary gonadotropin with macerated hog ovarian tissue in saline suspension. He found that the gonadotropic effect was definitely decreased by contact with ovary tissue if compared with controls which received the same two components separately. Contact with muscle slices had less effect, or none at all. He concludes that the gonads withdraw gonadotropic hormone from the blood. These experiments raise the problem of organ and species specificity of the protein hormones. It is probable that the above mentioned unconvincing results obtained by Seidlin by the action of hog ovary and hog liver on pregnant mare blood gonadotropin were due to the difference between the two species. Hormone action, it is true, is not bound to species specificity within the mammalian group; but protein hormone inactivation might be subject to other rules, since Seidlin succeeded in inactivating hog gonadotropin by hog ovarian tissue. Seidlin (1940) also injected guinea pigs with gonadotropic hormone prepared from hog pituitary glands. He found that the hormone can be recovered from the urine of male and female castrated guinea pigs, while it is present in much smaller quantities, if at all, in the urine of intact male and female guinea pigs similarly treated. This would suggest that inactivation of gonadotropic hormone takes place to a considerable extent in the gonads. Seidlin does not exclude the possibility, however, that castration leads to endogenous production of gonadotropic hormones in quantities not directly determinable but capable of synergistically enhancing the activity, and thus apparently increasing the recovery, of the hormone administered.

Robson and MacPherson (1940) found that castration had no effect on the rate of horse pituitary gonadotropin clearance from the blood of rabbits. It seemed, however, that the rate of disappearance of gonadotropin

from the blood was more rapid in pregnant than in non-pregnant rabbits. This may be accounted for by a special fixation of the hormone in the placentae of the animals as described by Hill and Parkes (1931). Robson and MacPherson (1940) further found that part of the horse pituitary gonadotropin disappeared—apparently through inactivation—when it was incubated for 12 hours with oxygenated blood at body temperature. It is possible that the bubbling of oxygen through the mixture was responsible for the loss of activity. This seems probable from the experiments of v. Euler and Zondek (1934).

### Summary

A specific organ for the inactivation of *chorionic gonadotropin in vitro* is unknown. Muscle, liver, spleen, ovary, testis, and kidney have proved to be devoid of any inactivating action *in vitro*. *In vivo* three organ systems take part in the process of inactivation, the reticulo-endothelial system, the spleen, and the kidney. In animals with the RES blocked as well as in splenectomized or nephrectomized animals chorionic gonadotropin is inactivated much more slowly than in normal animals. *Pregnant mare blood gonadotropin* seems to be slightly inactivated by the liver and the ovaries. *Pituitary gonadotropin* seems to be inactivated by the ovaries only.

### V. INTERVAL BETWEEN ADMINISTRATION OF GONADOTROPIC HORMONES AND APPEARANCE OF THE GONADOTROPIC REACTION IN THE ORGANISM

The first effect of chorionic gonadotropin on the ovary is *hyperemization* of the follicles (Zondek 1926, Zondek and Aschheim 1927). This may appear as early as 2 hours after injection. The reaction is surer after 6 hours, is at its peak after 12-24 hours and declines later. Its use for the diagnosis of pregnancy has often been mentioned, since the red ovary may easily be distinguished from its pale surroundings (Reipprich 1933, Zondek 1935, Walker and Walker 1938, Kelso 1940, Frank and Berman 1941, Salmon, Geist, Salmon, and Frank 1942, Kupperman, Greenblatt, and Noback 1943, Kaminester 1944, Zondek and Sulman 1945, Zondek, Sulman, and Black 1945).

Shortly after hyperemia *swelling* of the ovaries takes place (Zondek 1926, Smith 1926, Zondek and Aschheim 1927, Smith and Engle 1927). This has been specifically demonstrated for the pituitary gonadotropins rich in the so-called synergistic factor FSH (Evans, Simpson, and Austin 1933). In the rat the swelling is objectively demonstrated by increase of weight of the ovaries, the peak being obtained 24-48 hours after the injection. On the 4th or 5th day—when rats are usually opened to study the gonadotropic reactions—the ovary weight may have regressed to normal and the effect of the synergist can not be detected.

After hyperemia and swelling comes *ovulation* (Zondek and Aschheim 1926, 1927, 1928, Smith and Engle 1927). Bellerby (1929) studied the interval between intravenous injection of pituitary gonadotropin in estrus rabbits and ovulation and found the reaction to occur  $11\frac{1}{2}$  hours after injection. Similar results were reported by Friedman (1929). Fee and Parkes (1929) showed that mating of rabbits provokes a nervous stimulus affecting the pituitary anterior lobe which in turn induces gonadotropin production and secretion followed by ovulation. Hypophysectomy within 1 hour after copulation prevented ovulation, since elaboration of gonadotropin was not possible any more. Hypophysectomy at a later term, however, could not prevent ovulation which took its course about 10–12 hours after mating. Similar results have been reported by Smith and White (1931) in rabbits and by Hill and Parkes (1930, 1932) in ferrets. Parkes and Rowlands (1936) determined the time of gonadotropin formation and secretion in the rabbit pituitary after mating, by injection of gonadotropic antiserum. Up to 1 hour after copulation they were successful in inhibiting the follicular rupture by antigonadotropin. Later it was impossible to prevent ovulation which took its course within 15–20 hours. Similar experiments have been reported by de Fremery (1937) and Emmens (1940). Removal of a portion of the blood of the ovulating rabbit 1 hour after copulation did not prevent ovulation; Brambell and Parkes (1932) removed 30–40%, and McPhail, Parkes, and White (1933) even 50–60%.

In mice or rats, about 24 hours after subcutaneous injection of gonadotropin, ovulation takes place. This has been shown by Zondek and Aschheim (1926, 1927, 1928), Rowlands (1942), Rowlands and Williams (1943), and Burdick, Watson, Ciampa, and Ciampa (1943). Ovulation is easily recognized by 3 signs:

- (1) ruptured follicles discernible with the naked eye,
- (2) opaque swelling of the distal part of the tube which contains the shed eggs (Rowlands, 1942),
- (3) preparation and inspection of the tube under a dissecting microscope (Rowlands, 1942).

The next reaction to chorionic gonadotropins (FSH & LH) is the appearance of the *follicle hemorrhage* ("Blutpunkt" = blood dot, Zondek 1926). It may appear as early as 14 hours after intravenous injection of chorionic gonadotropin in rabbits (Friedman's modification of the A-Z pregnancy test) but it becomes a definite reaction only 24–48 hours after injection. Boehm (1941) used an ordinary zipper placed in a midline incision in rabbits for repeated examinations of the ovary. The first changes recognizable in the ovaries are small reddish-black spots which become noticeable as early as 10 hours after injection. The reaction reaches its height after 45–75 hours and the ovaries return to normal after 10–13 days. Nizza and

Berutti (1936) injected infantile rabbits intravenously with chorionic gonadotropin and transplanted one ovary 30–120 minutes later into the subcutis of normal rabbits. They claimed that the transplanted ovary reacted 48–56 hours later with blood dot formation in spite of being deprived of the protracted gonadotropin stimulus as early as 30–120 minutes after its first action. When they compared the scope of reaction in the transplanted ovary with that of the resting, they found quantitative—but not qualitative—differences. These interesting results, which have not yet been confirmed, are in full accordance with the results of Fee and Parkes (1929), Smith and White (1931), and Hill and Parkes (1932), who obtained ovulation in rabbits in spite of removing the gonadotropic source by hypophysectomy 1 hour after the start of its effect. Parkes and Rowlands (1936) giving antigonadotropic factor after mating, could prevent ovulation if antiserum was given up to 1 hour after copulation, but later on, ovulation could no longer be prevented. These results are at variance with those obtained by Zondek and Sulman (1937) with rats injected subcutaneously with chorionic gonadotropin. Up to 18 hours later the gonadotropic reaction could be prevented entirely by injection of anti-chorionic gonadotropin. This demonstrates that species differences and experimental techniques deserve special consideration. In mice subcutaneously injected with chorionic gonadotropin the blood dots appear 72 hours after the injection. This interval may be shortened by repeated intravenous, intracardial, intrauterine, or intraperineal injections of 10 IU up to 48 hours (Zondek and Sulman, unpublished results). We have also tried to shorten the period of blood dot formation in rabbits by increased dosage of chorionic gonadotropin but this has proved fruitless (Zondek, 1935). Rabbits injected intravenously with about 100 times the effective dose of chorionic gonadotropin (13,000 RU) did not show blood dots any earlier than the controls injected with threshold doses.

The appearance of the *follicle stimulating effect* has been studied by Frank and Salmon (1936/37). They injected immature female rats with gonadotropin from pregnancy urine, castrate urine, and pituitary anterior lobe and came to the following conclusion: "It appears that in some cases the immature rat ovary will respond to gonadotropic hormone with a follicle stimulating reaction as early as 26 hours after the first injection. This reaction becomes progressively more marked and reaches its maximum at approximately 64–72 hours after injections have begun."

*Vaginal estrus* appears in mice and rats not earlier than 60 hours after subcutaneous injection of chorionic gonadotropin and develops into full cornification by the 84th hour (Zondek, 1926). If pituitary gonadotropin is given, cornification is completed 12 hours earlier. Even the injection of estrone itself—the secondary hormone produced by the gonadotropins—

does not give earlier results. This indicates that the late reaction of the vagina to gonadotropins is due not to a delayed chain reaction but to the slow response of the target organ itself, the vagina. Our efforts to shorten this interval by increase of the hormone dosage have been unsuccessful, since high doses of gonadotropins evoke corpus luteum formation which paralyzes cornification through the mucifying effect of its hormone, progesterone.

*Corpora lutea* are developed in rabbits 84 hours after intravenous injection of gonadotropin, in mice 96 hours after subcutaneous injection, and in rats 108 hours after subcutaneous injection of chorionic gonadotropin (Zondek and Aschheim, 1926, 1927).

Corpus luteum formation has been followed up in detail by Deanesly, Fee, and Parkes (1930) in the rabbit. They found the first changes as early as 18 hours after copulation. At 24 hours lutein cells in process of growth became visible under the microscope, and at 36 hours the cells had obtained their normal large size. Corpus luteum formation discernible with the naked eye, appears, however, much later. Fels (1930) found the first histological changes in the rat ovary 40–50 hours after injection of chorionic gonadotropin. Experiments with a view to shortening these intervals by increase of dosage (Zondek, 1935) or intravenous injection (Zondek and Sulman, unpublished data) were without avail. Frank and Salmon (1936/37) found in the rat that luteinization begins after 64 hours and progresses steadily thereafter. In the majority of their cases luteinization reached its maximum at the end of 96 hours, only seldom later (120 hours). At 144 hours complete luteinization was found. They conclude that 3 factors influence luteinization, *viz.*, the method of gonadotropin extraction, the quantity of extract administered, and the time of observation.

Allen and Priest (1932), working on rabbits, and Goodman (1934), working on rats, transplanted the ovaries into the anterior chamber of the eyes of experimental animals. In both cases corpus luteum formation occurred at due term following the injection of chorionic gonadotropin.

### *Summary*

The gonadotropic reactions appearing after gonadotropin treatment occur at different time intervals depending upon the species of animal used, the kind of hormone administered and the route of application. Experiments to shorten these intervals have been in vain. A study into the mechanism of the gonadotropic reactions has shown that the delay encountered in their appearance is inherent chiefly in the target organ itself (which has to undergo a certain course of development) rather than in the slow action of the gonadotropins.

## VI. IS THE ACTIVITY OF THE GONADOTROPIC HORMONE DUE TO THE SUBSTANCE IN ITS ORIGINAL FORM?

The fact that a fixed interval is necessary for the development of the gonadotropic reactions raises the question whether the reaction of the ovary is brought about by chorionic gonadotropin in its *original* state, or whether this substance is altered on its way from the anterior pituitary gland to the ovary. In the latter case the primary reaction in the blood would be brought about by chorionic gonadotropin itself; while the specific effect on the ovarian cell would be produced by a quite different chemical substance ("chain-reaction"). To decide this question, chorionic gonadotropin was injected into the ovarian tissue (Zondek, 1935). In order to allow the injected hormone and the ovarian tissue to remain in contact for as long a time as possible, the large ovarian blood vessels were ligated and, in addition, the hilum of the ovary was clamped. Thus the ovary received its blood by only one small artery entering it at the upper extremity. The experiments showed that 50 per cent of the dose of chorionic gonadotropin which stimulates both ovaries by the intravenous method is sufficient to produce the gonadotropic reaction in the ovary into which it is injected. With a certain amount of reservation we may, therefore, conclude that chorionic gonadotropin in its original form, and not in a changed state, acts on the ovarian cells. If such a change had occurred, the intraovarian injection would have been unable to produce the reaction in the ovary, since most of the blood-flow to the ovary was cut off by ligation of the main vessels.

The effect of intraovarian injection of chorionic gonadotropin *in vitro* was studied by Zondek (1935) (in collaboration with H. v. Euler). After 5 hours' incubation at 37°C., there was sometimes an increase in the estrogen content of the follicle wall as compared with the control. These experiments were, however, not convincing.

As mentioned at the beginning, only a small part of the chorionic gonadotropin administered to the organism may be re-detected after a certain time. Now the question arises as to whether this tremendous loss occurs, due to the conversion of chorionic gonadotropin, in the course of its activity, into another substance, "*prolanoid*." We accepted, as a working hypothesis, that this substance must be closely related to chorionic gonadotropin, but that it is changed to such an extent that it is no longer active biologically, *i.e.*, not able to produce the gonadotropic reaction in the ovary, while on the other hand, it may inactivate "anti-prolan." The experimental procedure was described in 1940 and 1941 (Zondek), the hypothetical "*prolanoid*" being extracted after 30 hours from the carcasses of rats injected with prolan. From these experiments it was impossible to prove the existence of "*prolanoid*," a biologically inactive derivative of chorionic gonadotropin, which could inactivate "anti-prolan."

### Summary

The effect of chorionic gonadotropin is directly conferred upon the ovary without necessitating a chain reaction or an intermediate "prolanoid." This has been proven by intraovarial injection and by testing re-extracted chorionic gonadotropin (prolan) soon after its injection against titrated quantities of anti-prolan.

## VII. PRODUCTION OF THE SECONDARY SEX HORMONES IN THE OVARY

### a) Estrone Formation

Fels showed in 1930 that 36 hours after the subcutaneous injection of chorionic gonadotropin, the ovaries of rats may be *extirpated*, without preventing the appearance of the vaginal estrus reaction, 60-84 hours after the injection. The first histological changes in the ovaries appeared in 40 hours, generally after 50 hours. A further study was made in 1940 (Zondek) using the following technique: —infantile female rats were injected with varying doses of chorionic gonadotropin. After a certain period the animals were castrated and it was ascertained whether the estrus reaction appeared in spite of castration. The dosage varied from 2 to 30 IU of chorionic gonadotropin; the interval between chorionic gonadotropin administration and castration was from 24 to 36 hours. The experiments gave clear-cut results: 27 to 28 hours after chorionic gonadotropin administration the estrus reaction occurred although the ovaries had been removed. This means that it takes chorionic gonadotropin 27 hours to stimulate the production of estrogenic hormone and to release it into the blood stream.

If the ovaries of rats are subjected to examination 27 to 28 hours after administration of chorionic gonadotropin, no *morphological* changes are perceptible, which suggests, and this is of utmost interest, that estrogenic hormone has already been elaborated in the follicular cells and liberated into the blood stream before any changes are perceptible in the structure of the follicle. Estrogenic hormone is, therefore, effective in a premorphological phase. We have further tried to determine the length of time needed for estrone formation in the ovary by attempting to inactivate chorionic gonadotropin through anti-chorionic gonadotropin subsequently administered at different intervals.

In the preceding paragraphs it was mentioned that it is chorionic gonadotropin (prolan) *per se* which stimulates the ovary and not a derivative ("prolanoid") which may be inactivated by anti-chorionic gonadotropin ("anti-prolan"). An amount of anti-chorionic gonadotropin 10 times in excess of the required amount was injected in order to make sure that all the chorionic gonadotropin present should be inactivated immediately. The experiments shown in Table V demonstrate that 10 IU chorionic gonadotropin were inactivated by anti-chorionic gonadotropin after 26



hours and that the former had been active in the organism up to this time in its original form. After 28 hours (rat 3700), a certain amount of gonadotropic reaction becomes apparent (vaginal smear  $\pm$ ) suggesting that within this space of time estrogenic hormone has already been formed and has even become active to a certain degree. In another experiment (Table VI) larger doses of chorionic gonadotropin were given (30 IU) and the dose of anti-chorionic gonadotropin was 300 AU. This experiment, as the preceding one, demonstrated that after 26 hours even large doses of chorionic gonadotropin are completely inactivated by anti-chorionic gonadotropin and stimulation of the ovary is thus prevented. After 27 hours, however, irreversible stimulation of the ovary occurs resulting in vaginal estrus and corpus luteum formation.

TABLE V  
*Inactivation of Chorionic Gonadotropin by Anti-Gonadotropic Factor Injected Subsequently*

Rat	Dose of chorionic gonadotropin	Interval	Dose of anti-chorionic gonadotropin	Gonadotropic reaction (vaginal estrus)
<i>No.</i>	<i>IU</i>	<i>hours</i>	<i>AU*</i>	
3696	10	20	200	—
3697	10	22	200	—
3698	10	24	200	—
3699	10	26	200	—
3700	10	28	200	$\pm$
3701	10	Control		+
3702	10	Simultaneously	20	—

\* AU: 1 Anti-unit is the dose of anti-chorionic gonadotropin which neutralizes the effect of 1 IU of chorionic gonadotropin.

It is possible that the estrogen is produced in the ovary solely in the 26th hour after injection but it is also possible that the interval between the gonadotropin injection and the passage of estrogen into the circulation is taken up with the formation of an estrogen inactive precursor ("pro-estrogen") in the ovaries. The experiment recorded below was devised to test these points (Zondek and Sklow, 1942). An injection of chorionic gonadotropin into immature female rats was followed 18 hours later by the injection of sufficient anti-gonadotropin to neutralize the response in the rats' ovaries. This antihormone injection was followed in turn by a second gonadotropin injection, 20 hours after the first. If the estrogen produced is formed in the ovaries in the space of one hour only, namely 26 hours after the injection of gonadotropin, the estrus response of the rats should occur 20 hours later than it would have, after a single gonadotropin injection. If, however, pro-estrogen is already present in the

ovary when the antihormone is injected, this should be transformed into estrogen by the second gonadotropin injection without delaying the estrus response. This is actually what happened (Table VII). Thus estrogen is not produced solely during the 26th hour after the gonadotropin injection and it appears that production of a biologically inactive precursor does occur. It might be postulated that estrogen is being formed all along

TABLE VI

*Inactivation of Chorionic Gonadotropin by Anti-Gonadotropic Factor Injected Subsequently*

Rat	Dose of chorionic gonadotropin	Interval	Dose of anti-chorionic gonadotropin	Gonadotropic reaction		
				I Vaginal estrus	II Blood dot	III Cornus luteum
<i>n</i>	<i>IU</i>	<i>hours</i>	<i>AU</i>			
3727	30	24	300	—	—	
3728	30	25	300	—	—	—
3729	30	26	300	—	—	—
3730	30	27	300	+	—	+
3733	30	Control		+	—	+
3734	30	Simultaneously	40	—	—	—

TABLE VII

*Time of Estrus Response in Immature Rats Injected with Chorionic Gonadotropin Alone or With Anti-Gonadotropin*

Rat nos.	No. of rats	Injections		No. of rats in estrus		
		Dose	Time	60 hrs.	72 hrs.	84 hrs.
6482-506	25	1.5 IU	0 hr.	2	7	16
6507-16	10	2.5 IU	0 hr.	0	0	0
		5.0 AU	18 hrs.			
6517-41	25	2.5 IU	0 hr.	2	5	18
		5.0 AU	18 hrs.			
		7.5 IU	20 hrs.			

throughout the 26 hours after the gonadotropin injection and reaches a threshold concentration only at the end of this period. This is not, however, borne out by the results obtained with the second group of control rats. Subthreshold amounts of estrogen may be detected by a decrease in the number of leucocytes and the presence of epithelial or cornified cells in the vaginal smear—this was not found, the vaginal smears being completely negative in this group. We conclude therefore from the above that an inactive "pro-estrogen" is formed in the ovary during the 18 hours follow-

ing the injection of chorionic gonadotropin and that this "pro-estrogen" is converted into estrogen by the second gonadotropin injection.

The mechanism of estrogen formation in the ripening follicles has not yet been fully elucidated and it seems that in addition to the FSH the cooperation of the LH effect is also required in this process. This is suggested by the experiments of Greep, van Dyke, and Chow (1940) who, after injecting a very pure preparation of FSH into rats, brought evidence of stimulation of follicular growth without the production of estrogenic hormone. This was indicated by the lack of reaction on the part of the uterus and the vagina. Similar results were obtained by Fevold (1941). These recent results, which amply justify the original concept of Zondek and Aschheim concerning the dual nature of the gonadotropic hormones, are of far-reaching importance. They indicate that FSH stimulates morphological development, *i.e.*, follicle growth in the ovary, in the same way as spermatogenesis only is stimulated in the testes. On the other hand, LH stimulates hormonal activity, *viz.*, production of estrogen and progesterone in the ovary and—as is well known—testosterone and androstenedione production in Leydig's interstitial tissue of the testes.

It should be stressed that the latter conception is entirely new and not yet sufficiently established, but the analogy of the effects of FSH and LH on both the female and the male gonads is striking. An earlier suggestion of this possibility was made by Freed and Soskin (1937). On stimulation of rat ovaries with chorionic gonadotropin they found evidence of the formation of two estrogens, one from the theca and the other from the granulosa. The theca estrogen was found to be incomplete in its action since it did not induce endometrial proliferation. The granulosa estrogen was complete, resembling estrone in its action. This problem would seem to be worthy of further study.

#### b) Progesterone Formation

The data given in the preceding chapter for estrone are applicable—*mutatis mutandis*—to the formation of the corpus luteum and its hormone, progesterone. There is only one difference: If chorionic gonadotropin is injected intravenously into rabbits and 36 hours later the ovaries are extirpated, some layers of granulosa cells have already been formed, while there is still no decidual reaction in the uterine mucosa. With reference to the corpus luteum, therefore, morphological transformation precedes the liberation of progesterone. Thus, while the action of estrogenic hormone is premorphological, that of progesterone is *postmorphological* (Zondek, 1941).

#### Summary

Estrus may be prevented by an injection of anti-gonadotropin into rats 18 hours after the injection of chorionic gonadotropin, but if a second dose

of chorionic gonadotropin is injected 2 hours after the anti-serum, estrus occurs at the same time as it would have occurred had only the first injection been given. From this it is concluded that an inactive "pro-estrogen" is formed in the ovary (theca) during the 18 hours which elapse after the injection of chorionic gonadotropin. From the 18th to the 26th hour this "pro-estrogen" is transformed into the biologically active estrogenic hormone. In the following, 27th hour, it is released into the blood stream.

This follows from the simple fact that up to 26 hours after chorionic gonadotropin administration the gonadotropic hormone can be inactivated by anti-chorionic gonadotropin. Up to this moment chorionic gonadotropin must have been present in the organism without having had, as yet, a definite follicle-stimulating effect on the ovary. 27 to 28 hours after chorionic gonadotropin administration, even removal of the ovaries does not prevent the estrus reaction. During this period of time, therefore, chorionic gonadotropin has already initiated the elaboration of estrogenic hormone in the ovary and the latter substance has passed into the blood stream. Up to the point when estrogenic hormone production begins, chorionic gonadotropin is present in its original form, as it can be inactivated by anti-chorionic gonadotropin. We must conclude, that in the rat the presence of gonadotropin in its original form for 26 hours is a prerequisite for follicle stimulation and corpus luteum formation.

Progesterone formation obviously occurs later than estrone formation and also after the transformation of the granulosa cells becomes discernible under the microscope. This means that in the rabbit, progesterone formation begins at least 36 hours after the intravenous injection of gonadotropin. Whereas estrone is formed under the gonadotropic stimulus before anatomical changes are discernible in the follicle cells (premorphological hormone production), progesterone is only formed after the transformation takes place of some layers of follicle cells into granulosa (postmorphological hormone production).

In the light of recent investigations it seems probable that the effect of FSH is merely morphological (gametogenic), *i.e.*, causing follicle growth and spermatogenesis. On the other hand, LH seems to be the hormone-producing factor which stimulates the elaboration of estrogen and progesterone in the ovary and testosterone and androstenedione in the testes. This conception requires, however, further study and confirmation.

#### VIII. EFFECT OF MODE OF ADMINISTRATION OF GONADOTROPIC HORMONES UPON THE RESPONSE THERETO

The quantitative response of the organism to the gonadotropic hormones depends upon the rate of resorption. The slower the resorption, the stronger is the gonadotropic effect, since the loss of gonadotropin during the 24 hours' latent period amounts to 90% (*cf.* chapter II).

The influence of the route of administration is therefore more marked than that for the androgens, reviewed by Parkes and Emmens (1944). Studies of the following routes of administration of gonadotropic hormones are to be found in the literature:

1. intravenous
2. intramuscular
3. subcutaneous
4. intraperitoneal
5. intraovarial
6. peroral
7. rectal
8. vaginal
9. intraconjunctival
10. implantation of tissue and pellets.

1. *Intravenous Injection.* There is no doubt that the intravenous method of gonadotropin administration is superior to all others in shortening the latent period of reaction. This has been demonstrated by Friedman (1929). Zondek (1935) reports that follicle hemorrhage in the rabbit ovary occurs 16–24 hours after intravenous injection of 50–200 RU (= 10–40 IU) of chorionic gonadotropin. Bomskow (1939) comparing intravenous and subcutaneous administration found that in the rabbit follicle hemorrhage occurs as soon as 14 hours after intravenous administration (instead of 20 hours after subcutaneous injection). Mazer and Katz (1933) showed that the site of injection also influences the nature of the reaction (provided the same dose is given), intravenous injection producing mostly follicle hemorrhage and subcutaneous injection producing chiefly corpora lutea. Snyder and Wislocki (1931) compared the dose of chorionic gonadotropin necessary to evoke ovulation in rabbits by the intravenous, subcutaneous, and intraperitoneal routes. They found that 1 rabbit unit was the minimal effective dose by the intravenous route only, and that the minimal effective dose for subcutaneous and intraperitoneal administration was 40 times as great.

2. *Intramuscular Injection.* From the data available, it appears that the effects of intramuscular injection of gonadotropins equal those obtained by subcutaneous injection.

3. *Subcutaneous Injection.* Upon injecting minute amounts of gonadotropin subcutaneously into rats the following phenomena may be observed: Division of 1 RU (estrus unit) into several subcutaneous injections produces a smaller gonadotropic reaction than the administration of 1 RU in a single dose. With larger doses, however, this does not hold true. This may be explained by stating that, if a threshold dose (as 1 RU certainly is) is split up, several subthreshold doses are produced which cannot induce

the gonadotropic response in the ovary. This phenomenon was described for *pregnant mare blood gonadotropin* by Hamburger (1938) and for *chorionic gonadotropin* by Sulman and Sklow (1940). With regard to *pregnant mare blood gonadotropin* it was shown by Cole, Guilbert, and Goss (1932) that single large doses of this hormone are as efficacious upon the gonads as split doses. Leathem (1941) showed that a single injection—regardless of the route of administration—exhibited more potency than divided subcutaneous injections. In hypophysectomized rats corpora lutea formation resulted, in some animals, following intraperitoneal or intravenous injections but not after subcutaneous administration of the hormone. *Pituitary gonadotropins*, however, split up into many small doses give a stronger reaction than one single dose (Maxwell, 1934).

It is possible that the Maxwell effect of augmentation may be explained by the action of Evans' antagonist which is perhaps identical with the LH and with ICSH, the antigonadotropic effect of which becomes visible only when large amounts of it are resorbed quickly, *e.g.*, intravenously or intraperitoneally. Hence the gonadotropic reaction is weakened when one injection is given, thus favoring the action of the antagonist, and it is enhanced when many small injections of pituitary gonadotropin are given, since these maintain a continuous gonadotropic stimulus. This enhancement chiefly concerns the follicle-stimulating effect, the incidence of lutein changes being greatly diminished. Similar effects are also obtained by a variety of non-specific substances and extracts which slow up the resorption of the hormone from the site of injection. Some of these are: zinc sulfate (Maxwell, 1934), aluminium salts (Maxwell, 1934), copper sulfate (Fevold, Hisaw, Greep, 1936, 1937; Pfeiffer, 1937), ferrous sulfate, sodium sulfate, zinc chloride, potassium soap, agar-agar, gum tragacanth, gum arabic, glycogen, the Na-salts of the bile acids (Freud and Dingemanse, 1941), horse thyroid (Hellbaum, 1936), beef liver (Hellbaum, 1936), lemon juice (Hellbaum, 1936), milk (Hellbaum, 1936), casein (Saunders and Cole, 1936), egg albumin (Saunders and Cole, 1936), yeast extract (Fevold, Hisaw, Greep, 1936, 1937), blood of cattle (Casida, 1936), leucocyte concentrates (Kraatz, 1936), hemoglobin and heme (McShan and Meyer, 1937, Casida, Meyer, McShan, 1937), chlorophyll (Breneman, 1939; Leathem and Westphal, 1940), pregnant and normal mare serum (Saunders and Cole, 1936), tannic acid (Fevold, Hisaw, Hellbaum, Hertz, 1933; Bischoff, 1936), tungstic acid (Maxwell, 1934), formalin (Wallen-Lawrence, 1934, Maxwell, 1934), gelatine, glycerol (Zondek, Sulman, Sklow, unpublished, bismuth salts (Black, unpublished report from our laboratory).

Fluhmann (1933, 1938) has extensively studied the effect of administration of the various gonadotropins at different time intervals. The magnitude of the gonadotropic effect was judged from the weight increase of the

ovaries (and the uteri) of immature female rats treated with a standard amount of gonadotropin. This amount was spread over 5, 10, 15, and 20 days respectively. The results were as follows:

*chorionic gonadotropin*: maximal response when spread over 20 days,

minimal response when spread over 5 days;

*pregnant mare blood gonadotropin*: same response regardless of distribution;

*pituitary gonadotropin*: maximal response when spread over 5 days,

minimal response when spread over 20 days.

Fluhmann's findings also furnish a clear picture of the resorbability of the hormones. Pregnant mare blood gonadotropin appeared, however, to be a less effective ovarian stimulant than either sheep pituitary gonadotropin or chorionic gonadotropin, presumably of equal potency.

4. *Intraperitoneal Injection*. Evans, Korpi, Pencharz, and Simpson (1936) reported that "*pregnancy prolan*, which contains no antagonist, is distinctly less effective intraperitoneally than subcutaneously. Here the difference seems to be due merely to faster absorption by the intraperitoneal route with consequent increased rate of excretion by the kidney or destruction by the tissues." Sulman and Sklow (1940) have shown, however, that the intraperitoneal effect may equal the subcutaneous effect if the dose is split into 6 parts and distributed over 40 hours. If the minimum dose is given in 3 injections the intraperitoneal administration is about half as effective as the subcutaneous. If given in one injection by the intraperitoneal route the effectiveness is approximately 1/10 of that when given subcutaneously.

With regard to *pregnant mare blood gonadotropin* Evans, Korpi, Pencharz, and Simpson (1936) stated that its effectiveness is independent of the route of administration, when relatively large doses (24 RU) are distributed over 4 days, to rats. Pencharz (1939) has shown that in this case the relatively large initial dose provokes the main response and obscures minute discrepancies of reaction between the subcutaneous and the intraperitoneal route of administration. If, however, threshold doses were given over 3-12 days, the intraperitoneal route was 2-11 times superior to the subcutaneous route, especially in hypophysectomized rats. This has been confirmed by Leatham (1941).

*Pituitary gonadotropin* from various sources (cattle, sheep, pig) has been studied by Collip and Williamson (1936) who found the intraperitoneal method of administration to be much inferior to the subcutaneous one, if rats were injected 6 times during 6 days.

5. *Intraovarian Injection*. The method of intrafollicular and intraovarian injection in the rabbit was studied by Friedman (1932) who found that luteinization may be provoked by injection of threshold doses of chorionic gonadotropin into one ovary, while the other untreated ovary does not

undergo any changes. The presence of ripe follicle in the second ovary did not interfere with the luteinization of the injected ovary.

This method was extensively applied by Zondek in 1931 and 1935. By ligating the great blood vessels at the hilum ovarii and clamping off all the remaining vessels for half an hour, effective contact of the intraovarian injected chorionic gonadotropin with the ovary could be insured. The minimum dose of chorionic gonadotropin which is needed for the formation of follicle hemorrhages in both ovaries if injected intravenously, was also necessary to obtain the same magnitude of reaction in both ovaries on intraovarian injection. Half or two-thirds of this dose, however, was sufficient to induce the gonadotropic reaction in a single injected ovary.

6. *Peroral Administration.* Evans and Long (1921) using pituitary extracts and Zondek (1929) using chorionic gonadotropin studied the oral activity of gonadotropic hormones with negative results. Resuming these studies in 1935, Zondek found that, to obtain a gonadotropic reaction by this method, fairly high doses are needed and then only some of the animals react. Infantile female rats were fed 10–15 RU (estrus units) *chorionic gonadotropin* in milk. Some of them gave the anterior pituitary reactions I–III. In some cases up to 100 RU were needed to obtain vaginal estrus and up to 500 RU for luteinization. The response of some infantile female mice to as much as 1400 MU (estrus units) of chorionic gonadotropin was vaginal estrus only, without luteinization. The minimal effective dose was 20 MU. Thus, the effect of chorionic gonadotropin applied orally is only 10% in rats and 5% in mice, of the subcutaneous activity of the same dose.

Similar results have been reported by Reiss and Haurowitz (1929) and Dickens (1930) who found vaginal estrus in mice after the administration *per os* of 150 times or 40 times the parenteral dose. Janssen and Loeser (1931) needed 100-fold doses in mice. In rabbits they gave more than 30–40 times the intraperitoneal dose *per os* of chorionic gonadotropin without obtaining the gonadotropic effect. Huddleston and Whitehead (1931) also obtained positive results in rats when they gave high doses. Ehrhardt (1930) administered 10,000–16,000 U of chorionic gonadotropin to women. Resorption was obviously very poor since he did not find any of it in the blood and only in one case was FSH found in the urine. Friedman and Weinstein (1937) administered 8000–40,000 U of chorionic gonadotropin to men *per os*, of which nothing could be detected in the urine, besides which the gonadotropic effect was rather dubious.

If *anterior pituitary* extract is administered by the oral route gonadotropic effects are not observed unless 15–30 times the effective intraperitoneal dosage is given in rabbits (Lépine, 1931). Janssen and Loeser (1930) found 30–40 times the intraperitoneal dosage ineffective when given orally to rabbits. In 1931, however, they reported gonadotropic reaction



in rats protractedly treated with anterior pituitary gonadotropin; the dose needed was 100 times the intramuscular effective dose. Similar results were published as early as 1916 by Goetsch who fed infantile female rats for over 40 days with pituitary anterior lobe powder of unknown gonadotropin content. (The gonadotropic test reactions were then still unknown.) Gonadotropic stimulation (swelling of uteri and luteinization) was found when the rats were opened at a body weight of 90 g. and compared with the controls.

Oral studies of another kind were made by Sulman, Levy-Hochman, and Black (1945) by feeding infantile female rats with *chorionic gonadotropin tannate* by stomach tube. The doses ranged from 4–400 IU. Positive gonadotropic reactions (follicle maturation and luteinization) were observed in 14% of the cases only, namely those receiving doses of 50 IU. The results with free chorionic gonadotropin did not differ considerably from those obtained with chorionic gonadotropin tannate, thus confirming the variability of resorption and the uncertainty of gonadotropic reaction after oral administration.

7. *Rectal Administration.* Ehrhardt (1930) reported positive gonadotropic reaction I (vaginal estrus) in rats after rectal application of chorionic gonadotropin. Corpus luteum formation was not obtainable.

8. *Vaginal Administration.* Ehrhardt (1930) did not succeed in eliciting the gonadotropic reaction in rats by vaginal administration of chorionic gonadotropin. Lépine also reported (1931) negative results with pituitary gonadotropin given vaginally to rabbits.

9. *Intraconjunctival Administration.* Lépine (1931) tried the intraconjunctival administration of pituitary gonadotropin, in rabbits treated for 20 days, without result.

10. *Implantation of Tissue and Pellets.* Implantation of tissue as a precise method of hormonal tissue assay was first described by Zondek (1930) using detoxication with ether. It has been applied not only to endocrine glands but also to the diagnosis of mole or chorionepithelioma from curettage material. In positive cases as little as 0.02 g. may be sufficient to elicit the gonadotropic reaction in an infantile female mouse. The method was later applied to tissue suspensions by Neumann (1931) and to tissue powder by Janssen and Loeser (1931), Choay and Désaille-Merlhes (1937) and others.

The effects of *pellet* implantation were described in Chapters 1 and 2. Parkes (1942) prolonged the effect of chorionic gonadotropin up to 6 days by administration in cholesterol pellets. Sulman, Levy-Hochman, and Black (1945) using chorionic gonadotropin tannate pellets prolonged the effect up to 7.5 days. It is interesting to note that this prolongation of chorionic gonadotropin resorption does not result in an increased reaction of the ovaries as judged by their increase in weight.

### Summary

The peculiarities of the various methods of gonadotropin administration have been described. In summary, the effective doses by the various routes of administration may be listed in the following order starting with the most effective, designated as 1:—

intraovarial	1
intravenous	2 fold
intramuscular	2 fold
subcutaneous	2 fold
intraperitoneal	2-20 fold
peroral	30-200 fold.

The rectal route of administration is unpromising, the vaginal and conjunctival are ineffective. The pellet method is superior only as to the duration of the effect and not as to the degree of reaction obtained. The methods of achieving augmentation of the gonadotropic effect, especially of the FSH of the pituitary, are reviewed.

### IX. SYNERGISM BETWEEN THE ANTERIOR PITUITARY LOBE AND CHORIONIC GONADOTROPIN

The effects produced by chorionic gonadotropin necessitate the activating or synergistic cooperation of the anterior pituitary lobe. This puzzling fact may be seen by the incomplete reaction to chorionic gonadotropin in immature (suckling) animals and in hypophysectomized infantile and mature animals (Hill and Parkes, 1930, Reichert, Pencharz, Simpson, Meyer, and Evans, 1931, Evans, Meyer, and Simpson, 1932). Collip, Selye, and Thomson (1933) distinguish 3 phases of the gonad. reaction as seen in rats.

1. *1st-18th Day of Life.* The granulosa cells do not react to any kind of gonadotropic hormone. *Chorionic gonadotropin* causes a change of the theca cells to theca-lutein cells, whereas *pregnant mare blood gonadotropin* and *pituitary gonadotropin* are ineffective. The refractoriness of the reaction is inherent in the ovary itself, since it cannot be stimulated even after implantation unto adult female rats. In rabbits the refractory period of the ovary lasts until the 12th week of age (Snyder and Wislocki, 1931). Male rats do not respond to any gonadotropin.

2. *18th Day and Later, and Hypophysectomized Rats.* *Chorionic gonadotropin* evokes follicle stimulation and luteinization in infantile rats because of the interaction of their hypophyses. Chorionic gonadotropin evokes, however, theca luteinization in the hypophysectomized animal at any age and, in addition, continuous vaginal estrus in adult hypophysectomized rats. *Pregnant mare blood gonadotropin* evokes follicle stimulation only. *Pituitary extract* evokes follicle stimulation and luteinization, as do meno-

pause and male urine gonadotropin. Male hypophysectomized rats treated with chorionic gonadotropin show degeneration of the germinal epithelium, with marked overdevelopment of the interstitial tissue. Consequently the accessory sex organs do not atrophy.

3. *During Estrus of Mature Females or Hypophysectomized Mature Females Primed with FSH.* Here the ovary reacts fully to all gonadotropic hormones including chorionic gonadotropin.

The synergism between chorionic gonadotropin and the pituitary anterior lobe is an interesting fact, the mechanism of which has not yet been fully elucidated. The basic contribution to this mechanism was the discovery by Evans, Meyer, and Simpson (1931, 1932), Evans, Simpson, and Austin (1933), Evans, Pencharz, and Simpson (1934) that the effect of chorionic gonadotropin may be considerably enhanced by the addition of threshold doses of anterior pituitary lobe extracts. Even in hypophysectomized male and female rats gonad stimulation by chorionic gonadotropin is increased if practically ineffective doses of anterior pituitary gonadotropin are simultaneously administered (Collip, Selye, and Thomson, 1933, Leonard and Smith, 1934, Evans, Pencharz, and Simpson, 1934). The assumption of a "synergist" as a separate and distinct gonadotropic fraction of the pituitary as first postulated by Evans (1935) has since been revised, as Evans himself has shown that the properties of the synergist are contained in pure FSH fractions (Jensen, Simpson, Tolksdorf, and Evans, 1939, Evans, Simpson, Tolksdorf, and Jensen, 1939, Fraenkel-Conrat, Simpson, and Evans, 1940).

Based on experiments with hypophysectomized animals the view generally accepted to-day is that chorionic gonadotropin contains mainly LH and in order that it may take effect the cooperation of a "prohormone," which might be the FSH available in the anterior lobe, is required (Leonard, 1933, 1934, Fevold, Hisaw, Hellbaum, and Hertz, 1933, Fevold and Hisaw, 1934.) Pregnant mare blood gonadotropin, on the other hand, contains mostly FSH and hence its effect is more similar to that of the pituitary gonadotropin. The interstitial cell-stimulating factor (ICSH) first described by Evans, Simpson, and Pencharz (1937) was later shown by Evans, Simpson, Tolksdorf, and Jensen (1939) to be identical with the LH.

If large doses of chorionic gonadotropin are given to hypophysectomized animals the lack of FSH may be overcome, probably by the inclusion of minute amounts of FSH. This was shown to be the case by Reichert, Pencharz, Simpson, Meyer, and Evans (1932) who injected hypophysectomized rats with 400 U chorionic gonadotropin daily and by Hill and Parkes (1931) who obtained ovulation in hypophysectomized rabbits with high doses of chorionic gonadotropin. These authors also found that chorionic gonadotropin injected into pregnant intact or hypophysectomized rabbits was stored in the placenta of these animals and that extracts of

these placentae furnished powerful synergistic gonadotropins of the pituitary type. Extracts of uninjected rabbits' placentae were found to be completely inactive.

It seems that the synergism of the gonadotropins is not merely chemical, since it is known that lactating rats react like hypophysectomized rats to the application of chorionic gonadotropin (Bomskow, 1939). This would imply that the FSH of the anterior pituitary lobe is barred from entering the blood stream by prolactin or the other hormones participating in lactation. This does not, however, hold true in rats, the pituitary glands of which have been blocked by large doses of estrogenic hormone (Zondek, 1936, 1937, 1941). In these animals the secretion of gonadotropic and somatotropic hormones is impaired to such an extent that eunuchoid dwarfs result. The atrophic ovaries of these animals react, however, to the injection of chorionic gonadotropin, with follicle ripening and luteinization.

Similar relations were found in experiments with *thallium* (Buschke, Zondek, and Bermann, 1927). In animals fed small amounts of thallium, estrus is arrested. The ovaries of such animals contain follicles which are ready for rupture, corresponding in size and appearance to those found in estrus. Although anatomically they are perfect, they no longer produce estrogenic hormone. If, however, either anterior pituitary tissue is implanted into the animals, or chorionic gonadotropin is administered, estrogenic hormone production sets in and the animals again go into estrus. The explanation of this phenomenon may be that the anterior pituitary is blocked by thallium; but that this block is not complete, so that minute amounts of gonadotropic hormone, which suffice to maintain the anatomical intactness of the ovary, are allowed to pass into the blood stream. The dose of gonadotropic hormone necessary to bring about this effect (preservation dose) must be extremely small, certainly below 1 RU, since 1 RU is sufficient to initiate estrogenic hormone production in the theca cells, to liberate the latter hormone, and to produce estrus, none of which takes place in the thallium-fed animals. One RU does not suffice to initiate anatomical maturation of the follicle. As shown by the above experiments, the ovaries which are extirpated 27 hours after chorionic gonadotropin administration showed no signs of maturation of the follicle, although estrogenic hormone was already being produced in the theca cells and released into the blood stream. Production of estrogenic hormone in the theca cells, therefore, takes place at an earlier stage than enlargement (*i.e.*, maturation) of the follicle. The production dose initiating estrogenic hormone formation (1 RU), therefore, is smaller than that necessary to bring about follicular maturation with its typical biological picture (follicle fluid surrounding the cumulus oophorus).

As to quantitative considerations, the "ovarian preservation-dose"

is smaller than, and the "follicle maturation-dose" is larger than, the "hormone production-dose," which is equal to 1 RU.

### Summary

The gonadotropic effect of *chorionic gonadotropin* in the organism is based upon the cooperation of a gonadotropic factor furnished by the treated animal's own hypophysis. Without it, only a partial reaction takes place, characterized by

- (a) hypertrophy of the theca cells,
- (b) hypertrophy of the interstitial cells,
- (c) persistent estrus.

The cooperating factor of the anterior pituitary lobe, the so-called "synergist," is presumably the FSH, which is lacking in chorionic gonadotropin.

On the other hand, *pregnant mare blood gonadotropin* contains mostly LH, and *pituitary gonadotropin* contains FSH and LH in varying proportions depending on the species.

In view of the nature of the gonadotropic effect of chorionic gonadotropin 3 different doses may be distinguished. If we consider as 1 RU the minimal dose necessary for vaginal estrus production the following may be listed:

- (1) Ovarian preservation dose < 1 RU.
- (2) Hormone production dose = 1 RU.
- (3) Follicle maturation dose > 1 RU.

These differences are apparently due to different synergistic actions of the *pituitary gland* of the animal injected. The mechanism of this synergism, under different conditions, is not yet fully elucidated.

### X. REACTION OF DIFFERENT SPECIES TO VARIOUS GONADOTROPINS

It is interesting to note that the qualitative reactions of different species to gonadotropins vary. For instance, *follicle hemorrhage* (APR II) is seldom found in rats, guinea pigs, and in the muride *Microtus guentheri*, whereas in mice, rabbits, and in the muride *Meriones tristrami* it is the rule (unpublished results from our laboratory). An adult mouse never shows follicle hemorrhages in its ovary unless injected with gonadotropic hormone.

Quantitative differences in the response of different species to chorionic gonadotropin have been demonstrated by Zondek (1924, 1931). Whereas the body weight relations of the mouse, rat and rabbit are 1:5:200, the minimal units of chorionic gonadotropin for these animals follow the proportion 1:1/5:5. This shows, that as the weight of the species increases the sensitivity to chorionic gonadotropin also increases. This does not hold true, however, with regard to pregnant mare blood gonadotropin and pituitary gonadotropins, to which the mouse is more sensitive than the rat (Hamburger, 1933, 1934).

The following are some of the various reactions elicited by the different gonadotropins:

- (1) *Chorionic gonadotropin* is to a large extent ineffective with regard to the ovaries of hypophysectomized animals (Reichert, Pencharz, Simpson, Meyer, and Evans, 1931, 1932; Freud, 1933; Freud and de Jongh, 1933; Hamburger, 1936), rhesus monkeys (Allen, 1932, 1940; Johnson, 1935; Hamburger, 1936), guinea pigs (Loeb, 1932), and the muride *Microtus guentheri* (Zondek and Sulman, 1940); whereas *pregnant mare blood gonadotropin* (Engle and Hamburger, 1935), and *pituitary gonadotropins* (Westman, 1940), do stimulate them. In the monkey this reaction is obvious from the reddening and swelling of the sexual skin (Engle, 1930, 1933, 1934).
- (2) With regard to women and apes reports on the effect of the different gonadotropins on the ovary are contradictory. It is generally agreed that the gonadotropins induce hyperemization of the female genital tract as reported by Zondek, (1929, 1931, 1934/35, 1935). The morphological effect on the ovary is, however, inconstant and the data contradictory (Hamblen, 1939, Ryneerson, 1944). With *chorionic gonadotropin* follicle hemorrhage and luteinization (APR II and III) have been obtained in women by Zondek (1929, 1931, 1934/35, 1935); Mandelstamm and Tschaikowsky (1930); Geist (1933), and others. With *pregnant mare blood gonadotropin* ovulation has been produced in women by Davis and Koff (1938); Siegler (1940); Gray (1940); Kenny and Daley (1940); Griffith and McBride (1942). With *pituitary gonadotropin* no clear-cut results have been reported. Furthermore, many authors have reported entirely negative results from the treatment of women with the beforementioned 3 gonadotropins (cf. review by Davis and Hellbaum, 1943).
- (3) *Chorionic gonadotropin* does not stimulate the growth of the testes in the infantile cock, duck, pigeon, and canary, while *pregnant mare blood gonadotropin* (Hamburger, 1936) and *pituitary gonadotropin* (Riddle, 1931; Riddle and Polhemus, 1931; Schokaert, 1933; Evans and Simpson, 1934) does.
- (4) *Chorionic gonadotropin* stimulates only the Graafian follicles of the ovary but *pregnant mare blood gonadotropin* (Hamburger, 1934) and *pituitary gonadotropin* (Evans, 1935) stimulate the primordial follicles also. For this reason weight increase is limited in the infantile rat ovary up to 70 mg. following administration of large doses of chorionic gonadotropin but goes up to 250 mg. following pituitary gonadotropin injection (Evans and Simpson, 1929).
- (5) While the infantile rat ovary is much more sensitive to *chorionic gonadotropin* than is that of the infantile mouse (Zondek, 1929)—25

times more per gram of body weight—the sensitivity to *pregnant mare blood gonadotropin* and *pituitary gonadotropin* is more or less in direct proportion to the body weight (Hamburger, 1933, 1934).

- (6) The differences between the action of *chorionic gonadotropin* and *pituitary gonadotropin* in the rat have been compiled by de Fremery (1938) as follows:

<i>Pituitary gonadotropin</i>	<i>Chorionic gonadotropin</i>
Estrus scarce	Estrus strong and frequent
Weight increase of ovary parallel to dose	Weight increase of ovary brought about by high doses only
Uterus slightly enlarged	Uterus greatly enlarged
Preputial glands strongly developed	Preputial glands slightly developed
Corpora lutea small but numerous	Corpora lutea large but few
Vesica seminales slightly swollen	Vesica seminales greatly swollen

### Summary

The nature of the reactions of different species to gonadotropins varies. Quantitative differences as to the scope of the reaction and the minimal dose required indicate that larger animals are more sensitive to gonadotropins than small animals, judged on the basis of body weight.

The differences between chorionic and pituitary gonadotropins are manifest from the limited reactions of the ovaries in hypophysectomized animals, normal women, monkeys, guinea pigs, and other rodents injected with *chorionic gonadotropin*. In birds the testes respond to *pregnant mare blood gonadotropin* and *pituitary gonadotropin* but not to *chorionic gonadotropin*. In rats the difference between the various gonadotropins is indicated by the type of reaction of the ovarian follicles and corpora lutea, and by the response of the uterus, the preputial glands, and the vesica seminales.

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# The Role of Acetylcholine in the Mechanism of Nerve Activity

By DAVID NACHMANSOHN

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## I. INTRODUCTION

From the observations of Galvani and the subsequent controversy with Volta two essential facts of nerve and muscle physiology emerged: both structures are stimulated by electric current and, if active, generate electricity. During the last century the study of the electric changes during nerve activity constituted the best and in fact the only way open to scientists who were interested in the mechanism of nervous function. The rapid development of electric recording instruments led to great achievements in electrophysiology. But although electrical analysis is extremely helpful for the explanation of many phenomena of nerve activity, obviously the mere recording of the physical changes is not sufficient for a complete understanding of the mechanism involved in nervous function. Nerve activity like that of all cells requires energy. In a living cell chemical reactions are the only conceivable source of energy. The energy required for nerve activity is, however, so small that it was until recently below the range of measurement by physical as well as chemical methods. Not until 1926 when methods of a high degree of perfection were developed by A. V. Hill and his associates, was it possible to measure the heat production of the resting nerve and the increased heat production following stimulation (Downing, Gerard, Hill, 32). The observation of *heat production* as well as the measurement of the extra oxygen uptake (Gerard and Meyerhof, 49, Fenn, 43) brought experimental evidence for the assumption that nervous function is connected with chemical as well as electrical changes.

Heat production and oxygen uptake are, however, over-all reactions. The finer mechanism of the correlation between electrical phenomena and chemical reactions remained obscure (A. V. Hill, 57). In this review investi-

gations will be described which have led to some information about the relationship between the chemical reactions and electrical changes observed and about the chemical mechanism supplying energy for nerve activity. Since the space is limited it is impossible to discuss all observations in this field. Previous work, moreover, has been repeatedly reviewed. It is intended to describe here only those facts which are considered essential for the main problem.

The function of nerve cells to carry messages from one distant point of the organism to another offers three distinct problems: first, how a stimulus leads to an excitation, second, how an impulse thus initiated is transmitted along the nerve fiber, and third, how, at the nerve ending, this impulse is transmitted either to a second nerve cell or to the effector organ. Electrophysiologists, until recently, studied mainly the excitable properties of the nerve *fiber*. The results obtained by more than a century of intensive research form an integral part of physiology and need not be discussed here. Present concepts can be found in the books of Sherrington, Adrian, Gasser and Erlanger, Fulton, and in many reviews. Modern theories of excitation, the "excitatory disturbance," as Keith Lucas (73) called it, are discussed by A. V. Hill (59) and K. F. Bonhoeffer (14).

At the beginning of this century the conception evolved that a chemical substance might intervene in transmission of impulses from efferent nerve to effector cell. T. R. Elliot, in 1905, first suggested that sympathetic nerve endings might liberate a substance like adrenaline which would then act upon the cell. Elliot's suggestion was based on the very close similarity between the actions of adrenaline and sympathetic nerve stimulation. Although several other similar theories were proposed and several attempts made to test them, an observation of Otto Loewi was first to support the idea that a chemical substance transmits a nerve impulse from a nerve to an organ. When the vagus of a frog's heart was stimulated there appeared in the Ringer solution filling the heart a substance which, if transferred into another heart, reproduced the effect of vagus stimulation. Loewi concluded that the vagus acts on the heart muscle by the liberation of a substance which in its turn stimulates the heart muscle directly. He called the substance "vagus-stoff." It was later identified with acetylcholine (ACh). He also demonstrated that atropine does not decrease the liberation of the "vagus-stoff" but prevents its effect on the heart. The enhanced effect of vagus stimulation after treatment with eserine was explained by an inhibition of an enzyme destroying the vagus stoff (Otto Loewi, 68). These observations led to a great number of investigations, and evidence was accumulated supporting the view that all sympathetic and parasympathetic nerves act on the effector organ by virtue of chemical mediation. It is widely believed that acetylcholine is the transmitter substance at

parasympathetic nerve endings, whereas adrenaline or, according to Cannon and his associates, a related substance, sympathin, has the same function at the endings of sympathetic nerves (Rosenblueth, 124).

Loewi's work was confined to the autonomic nervous system. Kibjakow (63) and later Dale and his associates tried to extend this concept and suggested that acetylcholine might be the transmitter across ganglionic synapses and at neuromuscular junctions. Their evidence was based essentially on the same type of experiments as was applied in the case of the peripheral autonomic system: liberation of acetylcholine after stimulation of preganglionic fibers or motor nerves, stimulation of the sympathetic ganglion and the striated muscle by injection of small amounts of ACh, and potentiation of the effects of nerve stimulation by eserization. The results have been reviewed by Brown (16).

There remained, however, a great number of contradictions and difficulties well summarized by Eccles (33), and the evidence could not be considered as satisfactory (Fulton, 44, 45). Besides many other objections there were two main obstacles:—

(i) *The Time Factor*. For the transmission of the nerve impulse across ganglionic synapses or at neuromuscular junctions this factor is of primary importance and dominates all the aspects characteristic of this transmission. Autonomic nerves innervate slowly reacting cells. There it seemed less difficult to picture the nerve impulse acting by the release of a substance which in its turn stimulates the effector organ. Neurons and striated muscle fibers are very quickly reacting cells. Electrical signs of nerve activity indicate that the transmission of nerve impulses from neuron to neuron or from neuron to fibers of striated muscle occurs within a few milliseconds or within a fraction of a millisecond. Chemical reactions connected with this transmission must therefore occur with the same speed. In other words, if there is a compound responsible for the transmission of the impulse at those foci it must be released within this short period and must disappear with almost as great rapidity, *i.e.*, within the brief interval of the *refractory period*, for at the end of it the ganglion cell or muscle fiber is again fully responsive to any stimulus. Dale and his associates realized that the removal of the liberated ACh<sup>1</sup> must be rapid and admitted that this was one of the chief difficulties encountered by their theory (17). This time factor was also the main argument in Eccles' critical review against the theory of a transmitter function of ACh in transmitter processes of short duration. His main opposition was based on the argument that no evidence was available, at that time, that ACh can be removed with sufficiently high speed. According to all opinions, whether or not in favor

<sup>1</sup> Throughout this review *Acetylcholine* will be abbreviated to *ACh* and *Choline Esterase* to *Ch.E.*

of the transmitter function of ACh across synapses and neuromuscular junctions, it appeared essential to know whether the rate of ACh metabolism is as high as required by such a theory.

(ii) The second obstacle was even more fundamental. The "chemical transmitter theory" encountered strong opposition from the supporters of the "electrical theory." The terms are however misleading. The essential difference between the two opposite views was actually not the question of whether transmission of nerve impulses is electrical or chemical; it was the *question of whether the modes of conduction along fiber and across synapses differ fundamentally*. The work of Sherrington and his school indicates that the excitable properties of the central neurons are similar to the excitable properties of the peripheral nerves, *i.e.*, the axons (Fulton, 44). The problem has been scrutinized by Erlanger (35) in the Symposium on the Synapse, in 1939. Analyzing some of the peculiarities attributed to the synapses, namely latency, one-way transmission, repetition, temporal summation or facilitation, and transmission of the action potential across a non-conducting gap, he points out that all these phenomena can also be demonstrated on fibers. Gasser (47) arrives at a similar conclusion. The facts based on the electric signs of nerve activity thus make it unnecessary to assume, especially in view of similar time relations, that any condition exists at the synapse which differs, in any basic way, except quantitatively, from that found in the peripheral axon.

The development of Biology during the last twenty years has shown that much important information as to the function of cells may be obtained by the study of the enzyme mechanisms involved, since practically all cell activity is connected with enzymatic functions. Particularly in muscle physiology we have seen that study of the enzymatic reactions connected with contraction led to a real "revolution," according to an expression of A. V. Hill. Remarkable progress has been achieved in correlating physical changes with chemical reactions, although we are still far from a complete understanding of the mechanism of muscle contraction. Research in the field of vitamins and hormones has also been decisively influenced by findings which connected them with enzyme mechanisms. As Green (55) points out, it appears probable that all compounds which are active in minute amounts—"trace substances"—are correlated to enzyme systems, although at present this has been shown for only some of them.

Acetylcholine, supposedly a "neurohumoral transmitter," is a compound effective in amounts of the same order of magnitude as vitamins or hormones. It was to be expected that the study of the enzyme mechanisms connected with this ester, as well as other enzymes involved in nerve activity, should yield much information. In this way the electrical changes during the activity could eventually be correlated with chemical reactions.

The problem of the role which acetylcholine may have in the transmission of nerve impulses has been approached by Nachmansohn and his associates by investigations of the enzyme systems involved. This way, entirely different from that of Loewi, Cannon, Dale and their associates, has led to a change in the concept of the role of ACh in the mechanism of nerve activity. The new facts suggest that ACh is an essential link in generating the nerve action potential by its action on the surface membrane of the nerve. This new concept is based mainly on three lines of investigations: (i) the high rate of ACh metabolism in nerve indicated by the high concentration of *choline esterase*, (ii) the localisation of this enzyme at the neuronal surface, and (iii) the parallelism between voltage of the action potential and activity of the enzyme as is shown in electric organs.

## II. THE CONCENTRATION OF CHOLINE ESTERASE AT NEUROMUSCULAR JUNCTIONS AND SYNAPSES

### 1. Neuromuscular Junctions

The first essential problem which had to be studied was the question whether the rate at which ACh can be removed at synapses and at motor end plates is as high as is required by the theory of its transmitter function. The only probable mechanism for the removal of ACh with the required rapidity is a sufficiently high concentration of the enzyme which splits the active ester to its inactive components, acetic acid and choline.

Loewi and Navratil have shown that ACh can be enzymatically inactivated by heart extracts (69). This, however, is not surprising, since, as pointed out by Stedman, Stedman, and Easson (127), esterases are widely distributed in the animal organism and are known to hydrolyze a variety of esters. The important question was whether there is an enzyme which specifically hydrolyzes ACh. Stedman, Stedman, and Easson (l.c.) prepared from horse serum an enzyme which they considered to be an esterase specific for ACh and called it choline esterase. Later investigations do not support the assumption that the enzyme prepared by Stedman, *et al.* is really a specific choline esterase. There exists, however, an esterase which is specific for ACh. The specificity may be demonstrated by testing the action of an esterase on a number of substrates. In this way, a pattern may be obtained which makes it possible to distinguish the specific choline esterase from other esterases. The esterase in all nerve tissue is either exclusively or predominantly choline esterase. The enzyme is extremely stable. If kept at low temperature and at neutral pH, its activity remains unchanged for many months. The specificity of the enzyme as well as other properties will be discussed elsewhere (Nachmansohn and Rothenberg, 131; Nachmansohn, 102).

In living tissue ACh is a very unstable ester. If it is connected with



transmission of nerve impulses it will persist only for milliseconds at the site of action. It would, of course, be desirable to measure directly the rate of ACh release. No methods for such measurement are available at present. But the concentration of a specific and stable enzyme in a cell may be used as an indicator of the possible rate of metabolism of its substrate. One of the essential results of the work with isotopes, as pointed out in the review of Schoenheimer and Rittenberg (126), is the indication that no enzyme lies dormant during life, as some physiologists still believe, but is continuously active. Although the rate of activity may not always be maximal and excess of enzyme may be available, we have the right to assume that there does exist a relationship between the concentration of an enzyme and the possible rate of the metabolism of its substrate. If therefore a specific enzyme is highly concentrated in a cell, it appears reasonable to assume that its substrate may appear at a rate similar to that at which it can be removed. The possible rate of removal is therefore at present the best way to get information about the rate of ACh metabolism.

Striated muscles have a surprisingly low concentration of Ch. E. The rate of hydrolysis of ACh by muscle tissue is so slow that it would take about 300 seconds in frog, and 75 seconds in mammalian muscle to split 50 per cent of an amount of ACh which can account for its transmitter function (Marnay and Nachmansohn, 78, 82). Since the refractory period is about five milliseconds in the frog and two milliseconds in mammalian muscle, this time of hydrolysis is 60,000 times longer than the refractory period of frogs and 40,000 times longer than that of mammalian muscle. The conclusion drawn from these experiments was that the enzyme concentration at the end plates must be many thousand times higher than in the whole muscle if the ACh released there is to be split with the rapidity required by the transmitter theory.

Keith Lucas (72), in 1907, used the nerveless pelvic end of the frog sartorius for investigations on the excitability of pure muscle fibers. In order to test the possibility of a high concentration of the enzyme at the end plates the esterase power of this nerveless pelvic end of the frog sartorius has been compared with the part containing nerve endings. In the part containing nerve endings the concentration was about 300 per cent higher (Marnay and Nachmansohn, 79). Although the sciatic nerve contains more Ch. E. than does the muscle, the increase in concentration due to nerve fibers could be only very small. The strong increase found in the part containing nerve endings could be only attributed to a very high and peculiar concentration of the enzyme at these endings.

Subsequently, this was demonstrated in more detail. Pezard and May (118) investigated the distribution of nerve endings in the frog sartorius and found that only one-eighth of the pelvic end was really free from nerve

endings. The second fifth is particularly rich in nerve endings and then their frequency falls while the third fifth contains only half as many as does the second fifth.

When one-sixth to one-eighth of the pelvic end were compared with the second fifth and the third fifth, it was found that the second fifth of the sartorius contains about six times as much Ch. E. as the pelvic end, whereas the third fifth contains only three times as much enzyme (Marnay and Nachmansohn, 80, 83). We do not know the fraction which the volume of end plates constitutes in the whole muscle. According to rough estimations they probably occupy less than 1/5000 of the total volume, and we do not even know whether the enzyme is evenly distributed at the end plates or whether it may not be concentrated at a fraction of the end plate volume. If the enzyme were concentrated in 1/5000 of the whole volume, the figures obtained would indicate a concentration of 15000 to 30000 times higher than that in the nerveless muscle tissue. Although we cannot calculate how many thousand times Ch. E. is more concentrated at the end plates than in the surrounding fiber, we can estimate the approximate amount of ACh which can be split at a single nerve ending during the refractory period of the frog sartorius, that is in five milliseconds. As the difference of the rate of hydrolysis between the two parts may be attributed mainly to the esterase of nerve endings, we know the absolute amount which may be hydrolyzed there. The number of end plates of a sartorius of the size examined is, according to Pezard and May, about 600. This makes it possible to calculate that about  $2 \times 10^{-6}$   $\mu$ g. of ACh can be split at a single nerve ending during the refractory period. This amount corresponds to  $8 \times 10^9$  molecules of ACh.

These experiments furnished evidence that the concentration of Ch. E. at motor nerve endings is as high as is required by the assumption that ACh may be involved in the transmission of impulses across neuromuscular junctions. One great obstacle encountered by the theory, namely the time factor, was thus removed. Indeed, the very existence of such a peculiar biochemical system, the concentration of a specific enzyme at microscopic points many thousand times as high as in the surrounding medium, supports the assumption of the physiological significance of its substrate. It does not, however, indicate in which way the ester takes part in the transmission of the nerve impulse.

The experimental result of Marnay and Nachmansohn has been confirmed by Feng and Ting (42), but the conclusion has been contested by Clark, Raventos, Stedman, and Stedman (19), and later by Glick (54) and Little and Bennett (67). The paper of Clark, *et al.* has been discussed previously (Nachmansohn, 95, 96). A full discussion of all objections will be presented in another review (Nachmansohn, 102).

Evidence for a high concentration of Ch. E. at motor end plates can also be offered with mammalian muscle in a way analogous to that used in frog's sartorius. In the interior section of the gastrocnemius of guinea pigs the nerve spreads through nearly the whole muscle at one level only, situated in the midst of the muscle except for its entrance and its termination. In the middle third of this muscle all nerve fibers as well as the endings are situated in the middle zone. If this third is cut in slices with a freezing microtome, the slices near the upper and lower surfaces are practically free of nerve endings, whereas those of the middle zone contain a large number. Determinations of Ch. E. in these slices show a high concentration in the middle zone and low concentration in the two nerveless zones: whereas the *Q Ch. E.* [= mg. of ACh split by 100 mg. of tissue (fresh weight) in 60 min.] is 0.3 to 0.5 in the part free of nerves and motor end plates, the value is 2.0-3.0 in the slices of the middle zone (Couteaux and Nachmansohn, 23). Since the *Q Ch. E.* of the sciatic nerve is about 1.0, the same consideration can be applied as in frog's sartorius, *i.e.*, the *Q Ch. E.* values of the middle zone must be attributed not to the presence of nerve fibers but to the presence of a high concentration of enzyme at motor end plates. Further evidence for that is the fact that, following section of the sciatic nerve and the disappearance of nerve endings, the concentration of the enzyme in the middle zone remains high.

Another indication for the high concentration of Ch. E. at motor end plates is furnished by observations carried out on the perfused gastrocnemius of dogs (Marnay, Minz, Nachmansohn, 84). From the figures obtained it can be calculated that there exist two different rates at which the ester in the perfusion fluid is split: an extremely high rate in a very small volume and a slow rate in a very large volume. In connection with the experiments described above, this finding can be considered as an indication that in the living muscle the high rate of acetylcholine hydrolysis at motor end plates may actually occur.

## 2. Synapses

As at the end plates of striated muscle, Ch. E. is present in a high concentration at the synapses of sympathetic ganglia. Glick found that the concentration of Ch. E. in the superior cervical ganglion of the cat is not sufficiently high to split the amount of ACh liberated during the refractory period. Referring to the experiments of Marnay and Nachmansohn on the frog's sartorius, he suggested that there, too, the enzyme may be unevenly distributed and more concentrated around synapses (53).

It was, however, necessary to bring experimental evidence for this assumption. The sympathetic chain is practically free of a myelin sheath. It represents, therefore, nearly homogeneous grey matter, in which,

histologically, cells and nerve endings are concentrated in the ganglia. If the concentration of the enzyme in the ganglia of the sympathetic chain is compared with that in the fibers, the figures obtained for the ganglia are two to four times higher than for the fibers (Nachmansohn, 88, 96). The figures are, as in muscle tissue, mean values. The synapses occupy only an infinitely small fraction of the volume of the ganglion, although the inactive mass is much smaller than in muscle tissue. Therefore, if the difference is due even only partly to an increased concentration at the synaptic region, the concentration there must be very high in order to increase the mean value several times. The consideration is similar to that which must be applied in the case of striated muscle to the difference between the nerveless fraction and the part containing end plates, after having taken into account the value for the fibers.

The superior cervical ganglion of the cat is of special interest because more data is available here as to the possible role of ACh in synaptic transmission. According to the data reported, about  $1-3 \times 10^{11}$  molecules of ACh can be liberated in this ganglion by a maximal shock (Feldberg and Vartiainen, 38, MacIntosh, 74). Slightly lower values were found by Lorente de No, but they were still of the same order of magnitude (71). The concentration of Ch. E. has been determined in fibers and in ganglion. The Q Ch. E. values obtained were about 5.0 for the fibers, and 40 to 60 for the ganglion (Couteaux and Nachmansohn, 23). If we consider the difference between fiber and ganglion as being due to the higher concentration of the enzyme at the synaptic region, about 3-6 mg. of ACh can be split there during 60 minutes, or  $3-6 \times 10^{12}$  molecules of ACh during one millisecond. If the preganglionic fibers are cut, and have disappeared, the concentration of the enzyme has decreased by 60 per cent (Couteaux and Nachmansohn, 23). Only 1-2 per cent of this amount would be sufficient to split in one millisecond the amount of ACh which was found to be liberated by a maximal shock. The remaining amount of enzyme has to be considered as the amount present outside the preganglionic fibers.

When evidence was presented that ACh might be the transmitter of nerve impulses from motor nerve to striated muscle, or from neuron to neuron, many outstanding neurophysiologists envisaged the possibility of the same mechanism at the central synapses. Dale recalled, in his Harvey lecture in 1937, that Sherrington looks upon the transmission of excitation from a motor nerve ending to a voluntary muscle as probably furnishing a pattern of what happens at a central synapse (27). No experimental data were available at that time for such an assumption.

Investigations on the distribution and concentration of Ch. E. in the brain and spinal cord, carried out during the years 1937 and 1938, have shown that the same enzyme mechanism exists at central synapses as

that found at motor end plates and at ganglionic synapses (Nachmansohn, 86-92, 96). In the grey matter which contains the cell bodies and synapses, the concentration of Ch. E. is always high whereas it is comparatively low in the white matter. Great variations are found in the different parts of the brain, and the values vary also considerably in different species. A few examples are given in Table I (for more details see Nachmansohn, 96).

A most remarkable fact about these figures is the great constancy of the values for the same part and same species in contrast to the variations between the different parts and the different species. The values found by Nachmansohn have been confirmed by Pighini (119). The essential point is again that at central synapses amounts of ACh can be split in one milli-second which are of the same order of magnitude as those at ganglionic

TABLE I  
*QCh.E. of Different Brain Centers of Several Species*

	Rabbit	Dog	Ox	Man
1. Cortex.....	7	2-5	2-3	1.2
2. White matter (gr. hemisphere) .....		0.3	0.2	
3. Nucleus caudatus.....	57	57-59	40-43	30
4. Nucleus lentiformis.....			69	46
5. Cerebellum.....	9-10	12-13	2-3	8
6. Thalamus opticus.....	12	6	5	3
7. Pons.....	13	7-8		6
8. Tub. quadrigem. ant.....	25	14	10-13	6
9. Tub. quadrigem. post.....	13	5	4	3
10. Retina.....		15	16	

synapses. The superior cervical ganglion of the cat is very rich in cells and synapses; according to Billingsley and Ranson (13) the figure is about 100,000. If the same frequency of cells and synapses is assumed per unit of weight of the brain centers and the same amount of ACh released per cell, one arrives at figures which indicate a large excess of enzyme even if only half of the enzyme is present outside the nerve endings. The very existence of such a specific enzymatic system at all these foci strongly supports the view that the substrate there has the same function, and is consistent with the view of Sherrington that the mechanism of transmission is essentially similar at all synapses.

### *3. Choline Esterase During Embryonic Development*

If ACh is essential for transmission of nerve impulses across neuromuscular junctions and across synapses, Ch. E. should be present in high concentration at a very early stage of development, actually at the time

when nerve impulses begin to be transmitted at those foci. Such a relationship between enzyme concentration and function during embryonic development has been demonstrated in many different ways. A full description of the results achieved will be found elsewhere (Nachmansohn, 1952), but two examples may be given here as illustration.

In the muscle of chick embryos the concentration of Ch. E. increases rapidly to high values during incubation (Nachmansohn, 1955). The Q Ch. E.

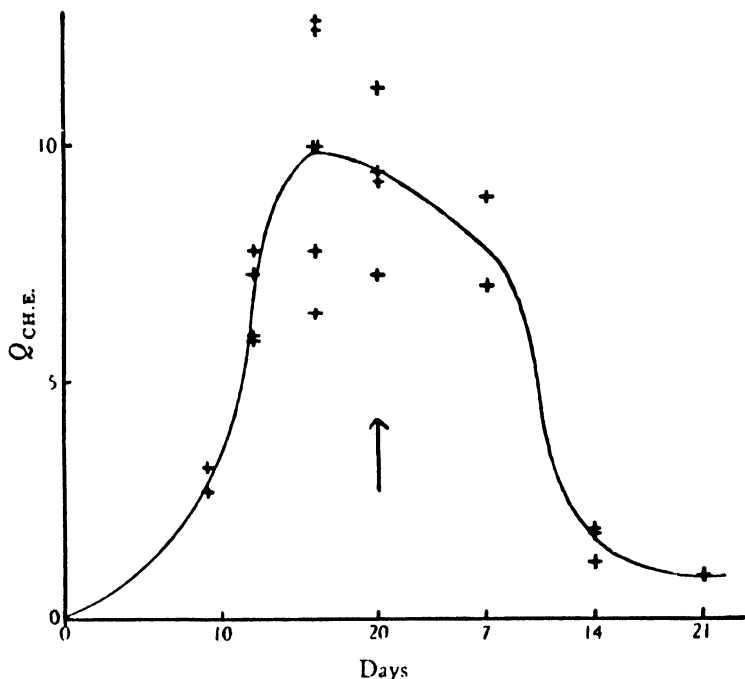


Fig. 1. Course of QCh.E. During Growth in Breast Muscle of Chicken  
Abscissae: Days of incubation and after hatching. The arrow marks the day of hatching. Ordinates: QCh.E.

of breast muscle, for example, was found to be 9.4 on the average at hatching, varying from 7.3 to 11.3. On the sixteenth day of incubation it was even slightly higher: 9.9 on the average, varying from 7.8 to 12.7. After hatching the values go down, the Q Ch. E. three weeks later being only 0.9 (see Fig. 1). The Q Ch. E. of fowl muscle is 0.4 to 0.5. The frequency of nervous elements and end plates is much higher in embryonic muscle than that in adults owing to the smallness of the muscle fiber. The observations of Couteaux (22) indicate that the end plates reach nearly their full

size at birth, so that while the muscle fibers of an embryo are small the end plates in a given weight of muscle are relatively numerous. Later the fiber grows, and the number of end plates per unit of weight decreases correspondingly. The fact that the Q Ch. E. is so high at the very stage of development when the number of end plates per unit of weight is high supports the view that there is a high concentration of Ch. E. at the end plates at that early stage.

Experiments carried out on sheep foetuses (Nachmansohn, 99) may be quoted as another example. The different centers of the central nervous system do not develop at an equal rate. During recent years this problem has been investigated by Barcroft and Barron (8) in connection with the movements and reflexes of sheep foetuses. Their observations offer evidence for the early development of spinal reflexes and of the relatively delayed period at which the brain enters into action. The times at which the different centers begin to function, according to the observations of Barcroft and Barron, coincide with the appearance of a high concentration of choline esterase: this concentration is high in the spinal cord at a very early age of the sheep foetus, but low at that time in the different brain centers; there it rises to high values only during the last weeks before birth.

The experiments so far described remove one main difficulty,—the question whether or not ACh can be metabolized at the high speed required. But there remains the other objection mentioned above, namely that the facts based on the electrical signs of nerve activity make unnecessary the assumption of a fundamental difference between conduction along axon and transmission across synapses. Although the electrical signs of nerve activity are not sufficient to explain the mechanism of nervous action, no conclusion drawn from observations with chemical or pharmacological methods should be in contradiction to that based on observations with the much more sensitive electric recording instruments.

The observations described in the following chapter show that Ch. E. is highly concentrated everywhere at the neuronal surface. There is only a quantitative difference between axon and synapse, the enzyme concentration being higher at the synapse where the neuronal surface increases due to the extensive end-arborization. This is consistent with the view that ACh may have the same role in the transmission of the nerve impulse along the axon as across the synapse; and that the difference may be only a quantitative one.

### III. LOCALISATION OF CHOLINE ESTERASE AT THE NEURONAL SURFACE

The concentration of Ch. E. is high in all nerve fibers but rises still higher in the synaptic region. This is particularly conspicuous in non-myelinated fibers. In the sympathetic chain of dogs, the values found for

the fibers varied from 3.3 to 7.7, in the ganglia from 11.1 to 19.1 (Nachmansohn, 88). In the abdominal chain of the lobster (*Homarus vulgaris*) which represents central nervous system, the Q Ch. E. values are as high as 5 to 15 in the fibers, rising to values between 18 and 30 at the points where the synapses are located. The Q Ch. E. values of myelinated fibers are somewhat lower, between 1 and 3. In the frog sciatic a value of 0.8 was found at 18° C., in dogs' sciatic the value is about 1 at 37° C. In dorsal roots of the dog the mean value found was 1.4, in the ventral roots 2.9. The optic nerve of the dog has a Q Ch. E. of 1 to 2. All these values are of the same order of magnitude even if compared with the non-myelinated sympathetic fibers. The myelin sheath seems to have only negligible amounts of enzyme. All white matter in brain and spinal cord has very low values. If during embryonic development in the spinal cord of sheep fetuses the myelin sheath appears, the value of Q Ch. E. drops (Nachmansohn, 99). According to Donaldson and Hoke (31) the myelin sheath forms 50% of the axon. Since in these 50% the amount of enzyme is negligible, the figures obtained have to be doubled to arrive at the concentration of the enzyme in the axon, and therefore the values are of the same order of magnitude as in the non-myelinated fibers of the sympathetic chain. But even then we do not arrive at the real concentration—in myelinated as well as in non-myelinated fibers—since this could be calculated only if the enzyme were evenly distributed in the axon, which as will be shown, is not the case.

The spinal ganglion is of special interest since it contains cell bodies but no synapses. The Q Ch. E. here is 2 to 3 times higher than that of the dorsal roots. Any quantitative interpretation of this figure, as compared with that of the dorsal roots, is complicated because the ganglion contains comparatively small amounts of myelin.

The high concentration of the enzyme in all nerve fibers suggests that ACh metabolism differs only quantitatively between fiber and synapse, and that its significance may not be limited to the nerve endings. Consistent with this view is the finding of Lorente de No (71) that ACh is released by ganglion cells after impulses have passed in which no synaptic transmission is involved, and in peripheral nerves. The release of ACh from "cholinergic" fibers following stimulation has been confirmed by Lissak (66).

In the experiments on the superior cervical ganglion it was observed that following section of the pre-ganglionic nerve fibers the Q Ch. E. of the ganglion falls from 40–60 to 20–25, during the first ten days. This is the time when the nerve fibers disappear. Thereafter the enzyme concentration remains constant for many weeks. The decrease is parallel to the time of the disappearance of the pre-ganglionic fibers and it is therefore reason-



able to assume that the enzyme which disappears is located inside the fiber. Since the Q Ch. E. of the pre-ganglionic fiber is only 5, the figures obtained indicate that the fibers inside the ganglion have a higher concentration of Ch. E. than before it enters the ganglion. These observations led Cou-teaux and Nachmansohn (23) to the conclusion that the strong increase of enzyme concentration in the pre-ganglionic fibers towards their endings may be connected with the increase of surface occurring inside the ganglion by the extensive end-arborization of the pre-ganglionic fibers. The experiments suggest that the enzyme may be more highly concentrated at or near the neuronal surface and that the high concentration of the enzyme inside the ganglion has to be referred partly at least to the increase of surface.

Direct evidence for the localization of Ch. E. at the neuronal surface has been offered with experiments on the giant axon of the squid (*Loligo pealii* Leseuer). This axon is today familiar to biologists, thanks to the brilliant work of Young and Schmitt and their associates (10, 130). It is of enormous size. From squids of average size one readily obtains axons 0.4–0.6 mm. in diameter and 60–70 mm. long. These afford an excellent opportunity for a study of single units. It is possible to extrude the axoplasm from the axon and to study its compounds and enzymes separately from those in the envelope. The envelope is mostly connective tissue, but attached to it there are two membranes, each of them only a few microns thick. Ch. E. is localized practically completely in the sheath. The esterase activity of the axoplasm is negligible (Boell and Nachmansohn, 15). At least 80–90 per cent of the sheath is connective tissue. The values of Q Ch. E. found have therefore to be multiplied by at least 5 to 10 times. But even then the values will be minimum values; the enzyme concentration may be still much higher than the activity per unit of tissue weight indicates, because it may be localized in a small fraction of the remaining tissue. The experiments thus support the previous results that a high concentration of Ch. E. exists everywhere at the neuronal surface. Consequently the figures given for the fibers do not indicate the real enzyme concentration since at least 90 per cent, and probably much more, is inactive tissue. It appears probable that the enzyme is located, as in the giant axon, in a layer of a few microns only. Since the difference between axon and synapse is in any case slight, the enzyme concentration being only four to five times higher in the synapse, the figures indicate that at the surface membrane of nerves considerable amounts of ACh may be metabolized in milliseconds.

It is important to know whether this localization of Ch. E. is specific. Studies on the distribution of other essential enzymatic systems known to be involved in nerve metabolism have been initiated (Nachmansohn,

Steinbach 105); Nachmansohn, Steinbach, Machado, Spiegelman, 106). It has been found that the bulk of the respiratory enzymes are localized in the axoplasm. About 90% of succinic dehydrogenase, widely considered as an important link in respiration (Szent-Györgyi, 128), is present in the axoplasm. The succinic oxidizing system has a similar distribution. The difference in the concentration of cytochrome oxidase between sheath and axoplasm is even more pronounced. The distribution of this enzyme is of particular interest since there is general agreement that the first activation of oxygen of physiological significance in living cells occurs through the cytochrome oxidase systems. The findings show that the rate of respiration is low at or near the neuronal surface. The rate of  $O_2$  uptake is in absolute values low even in the axoplasm as compared with other cells. Although oxidation must be the ultimate energy source for the action potential, the experiments are further evidence against the assumption of any direct or immediate connection between oxidation and action potential.

Bioelectric phenomena are, as has been for a long time generally accepted, surface phenomena. Experimental evidence for this was recently offered by Hodgkin and Huxley (60) and Curtis and Cole (26) with observations on the giant axon of the squid. The high rate of ACh metabolism at the neuronal surface makes possible the assumption that the ester is connected with the electric changes during activity. This assumption obtains further support from experiments on the electric organs described in the following chapter, in which a close correlation could be established between the voltage of the nerve action potential and the activity of the enzyme.

#### IV. PARALLELISM BETWEEN VOLTAGE AND ENZYME ACTIVITY IN THE ELECTRIC ORGAN

Twenty years before Galvani's discoveries, in 1772, Welsh demonstrated before the Royal Society in London that the shock of certain fishes, known since ancient days, was an electric discharge. It was the first evidence of animal electricity. When Galvani's observations made it clear that electricity is a common property of nerves and muscles, physiologists became interested in these organs. Galvani himself worked in the last year of his life on electric fish organs, and many outstanding physiologists of the last century, especially DuBois Reymond, made extensive investigations on these organs.

There are three species known with powerful electric organs and several others with weak electric organs. The most powerful species is the *Electrophorus electricus* (Linnaeus), the so-called "electric eel," which occurs in the Amazon in Brazil. The maximal discharge in medium sized specimens is on the average 400–600 volts, but may be in some individuals more than 800 volts. Another powerful species is the *Malapterurus*, which is found in

the Nile in Egypt. The maximal discharge can be 400 volts and even more. A more common species occurring in different parts of the world is the *Torpedo*. A particularly large species can be found occasionally at the east coast of this country particularly in the water around Cape Cod: the *Gymnotorpedo occidentalis*, first described by Storer in 1848. The discharge of the most common species of *Torpedo* is generally 30–70 volts, but in the large *Gymnotorpedo*, as has been measured by Amberson and Edwards, it is 150–200 volts (see Nachmansohn and B. Meyerhof, 103). A full description can be found in many handbooks and textbooks (Biedermann, 12, Rosenberg, 123). The electric organs, except those of *Malapterurus*, are phylogenetically evolved from striated muscle, as was shown by Babuchin in 1870. The muscular part has, however, completely disappeared in the strong electric organs. The organs of the different species all have a similar structure: they are formed by columns which are subdivided into compartments and in each compartment is an electric plate or disk. They resemble a Voltaic pile; Volta discovered this analogy and called his pile “an artificial electric organ.” But there is an important difference: if two ends of the Voltaic pile are connected there is an automatic flow of current. The discharge of the electric organ is a voluntary act of the fish, regulated by the central nervous system. On the basis of this difference, Berzelius arrived at the logical, but for his time most remarkable, conclusion that this discharge cannot be a physical process but must be a chemical process. In his textbook of chemistry he described, in 1817, the electric organs under the title: “Electricity elicited by an organic chemical process.”

The histological structure of the electric plates differs in detail in the various groups, but all present general features of resemblance. Only one side of the plate is innervated. That side becomes negative during the discharge whereas the opposite side becomes positive. The multinucleated protoplasm of the plates is homologous with the protoplasm of the motor end plate. For this reason and in view of its origin the organ is referred to as an accumulation of “modified motor end plates.”

The electricity of the discharge does not differ in principle from that of ordinary nerves. It is only the arrangement in series by which these organs are distinguished, and by which the great electromotive force is obtained. The voltage of the single plate varies in the different species between 50 and 150 millivolts, that is, the same order of magnitude as that of the action potential in ordinary nerves. It was already recognized by the physiologists of the last century that the discharge is identical with the nerve action potential. The reasons have been well summarized in 1898 by Gotch in Schaefer's Textbook of Physiology (Vol. II, p. 590). There are very serious objections to comparing it with muscle electricity since no muscular elements remain in the strong electric organs and since in the

electric organs of *Malapterurus glandular*, not muscular, elements have been used in the evolution of the organs. Thus there remain only two possibilities: an electricity either "*sui generis*" or based on the same process which occurs during nerve activity. The first possibility can be practically excluded:

"The views that the disc is an excitable structure responding to nervous impulses which reach it through its nerves is contradicted by all experimental observations. . . . There is no evidence that the discs can be excited apart from their contained nerves; all agents which annul the excitability of the electrical nerves annul, at the same time, the response of the organ to electric currents which traverse its substance. One of the most suggestive facts bearing on this point is the complete inexcitability of an organ when deprived of its nerves through nerve degeneration in consequence of previous section. Direct excitation of the organ is direct only in appearance, not in reality. . . . The only excitable structures in the organ are thus the nerves, and their final terminations, and since these are known to be the seat of electrical changes during activity, it seems more reasonable to presume that the organ change is in reality closely related to the production of molecular disturbances in its contained nerves."

Thus Gotch arrives at the conclusion that "the primary cause of the organ shock is in all probability a change identical in nature with that producing the electromotor phenomena of nerves." This is also the view of A. V. Hill (57):

"there is no reason to think that the shock given by the electric organ is, in any fundamental sense, different from the action current of nerve. In the former the elements are in series, in the latter in parallel: the nature, however, of their activity is almost certainly the same."

For the understanding of the mechanism of the discharge the time factor is again of great importance. In a single second 100-200 discharges can be obtained in the strong electric organs. Latency, duration of the discharge and refractory period are of the same order of magnitude as in the nerve action potential. When, in 1937, evidence was offered for the high concentration of Ch. E. at motor end plates, Nachmansohn envisaged the possibility of a similar mechanism in the electric organ in view of the analogy, and Marnay, on his suggestion, determined Ch. E. in the electric organ of *Torpedo* (77). For if a polarizing or depolarizing substance is responsible for the discharge, appearance and disappearance of the substance has to occur within milliseconds. In other words, for the assumption that ACh might generate the discharge the existence of a most powerful esterase system had to be postulated. The concentration of Ch. E. found in the *Torpedo marmorata* is remarkably high: one gram of electric organ splits 1.5-3.0 g. of ACh in 60 minutes corresponding to a Q Ch. E. of 150-300. Electric organs are highly specialized in their function. The

existence of such high enzyme concentration appeared particularly significant in view of the high water- and low protein-content of these organs: 92% of the organ is water and only slightly more than 2% proteins. The concentration of the enzyme is of the same order of magnitude as that estimated previously for motor end plates of muscle. The essential point here again is the fact that in these organs considerable amounts of ACh can be split during the discharge which is of the order of a few milliseconds. As the whole organ of *Torpedo* weighs 100–200 g., from 200 to 500 g. of ACh can be split in 60 minutes by 1 organ or 50–100  $\mu$ g. in one millisecond. This makes possible and in fact probable the assumption that ACh is closely connected with the discharge.

In the following year, 1938, Nachmansohn determined the concentration of Ch. E. in the weak electric organ of the common ray (*Raja undulata*), and in one medium-sized specimen of an electric eel.<sup>2</sup> The Q Ch. E. in the weak electric organ of the ray varied between 3 and 10. In two determinations made on the electric organ of *Electrophorus electricus*, the Q Ch. E. values found were 90 and 150.

If, in the three species, number of plates per centimeter and voltage per centimeter are compared with the concentration of the enzyme, a close parallelism is obtained (Nachmansohn, 97). This is further support for the concept that ACh is connected with the discharge, for obviously the higher the voltage per centimeter, the higher is the amount of ACh released per gram of tissue and consequently the higher has to be the concentration of Ch. E. for removing the ACh released.

Auger and Fessard (3–5) analyzing the latency and form of the discharge obtained on isolated prisms of the organ with electrical stimulation confirmed the conclusions of most previous investigators, *i.e.*, that excitation of the organ was always indirect (see also the above mentioned discussion of Gotch). They also confirmed Garten's observation that following section of the nerve direct and indirect excitability disappears simultaneously. In view of the findings of Marnay and Nachmansohn, they considered the possibility that the discharge may be produced by the release of a polarizing or depolarizing substance such as ACh, since the main difficulty for such an assumption was removed by the evidence of the high Ch. E. concentration. This view was also consistent with results which they obtained in their experiments on the effects of some drugs on the discharge.

It appeared desirable to test further the role of ACh in eliciting the discharge. Fessard and Nachmansohn decided to study (1) whether ACh

<sup>2</sup> This specimen which was the only one available died in October 1938 in the Institut Oceanographique in Paris, and the determinations were made in the Laboratoire de Physiologie Generale de la Sorbonne on the day following death.

is present in electric tissue, (2) whether it is released during the discharge and appears in the perfusion fluid, and (3) whether ACh, if injected, causes potential changes. In the summer of 1939, they investigated these questions at Arcachon, France, and were joined, on their invitation, by W. Feldberg. The following results were obtained on *Torpedo marmorata* (Feldberg, Fessard, Nachmansohn, 39): (1) The electric tissue contains about 50 to 100  $\mu\text{g.}$  of ACh per gram fresh tissue. (2) If the quiescent organ is perfused with eserinizied salt solution, no ACh is detectable in the perfusion fluid. During stimulation the ester appears in the perfusate. (3) Arterial injections of ACh (10–200  $\mu\text{g.}$ ) into the perfused organ caused potential changes in the same direction as those of the discharge. The maximal variation after injection of 200  $\mu\text{g.}$  of ACh amounted to about 0.7 mV, and after 20  $\mu\text{g.}$  to less than 0.1 mV. 5  $\mu\text{g.}$  were without effect. But if eserine was added to the perfusion fluid, the effects were greatly enhanced: with 2.5  $\mu\text{g.}$  of ACh the potential change was 0.5 mV, with 10  $\mu\text{g.}$  greater than 3 mV.<sup>3</sup>

Thus the experiments brought further support for the concept that ACh is connected with the discharge, as was suggested by the experiments of Marnay and Nachmansohn on the high concentration of Ch. E. and the variations in the different species, and by the different observations of Auger and Fessard on the electric aspects of the discharge.

The parallelism between voltage per centimeter, electric plates per centimeter, and concentration of Ch. E. if the different species are compared, is only approximate. The electrical and histological values were average values taken from the literature. A detailed study of the enzyme concentration in relation to the electromotive force appeared necessary. The *Electrophorus electricus* offers particularly favorable material; it can be kept for a long time in captivity, and therefore extensive and careful measurements are possible. Great variations of Ch. E. concentration occur in the organs of this species. The enzyme concentration decreases from head to tail end of the organ in an S-shaped curve, so does the voltage per centimeter and the number of plates per centimeter (Nachmansohn, Coates, Cox, 104; Cox, *et al.*, 24. Moreover, the voltage varies greatly between specimens of different size; the voltage per centimeter and the number of plates is much higher in small specimens and the variations more marked than in the large specimens.

The electrical values—voltage, amperage, resistance—have been recorded on a variety of specimens at different sections of the electric organ, and at the same sections enzyme determinations and histological preparations were made.

<sup>3</sup> A more detailed description of these experiments has been published independently by W. Feldberg: Feldberg, W., and Fessard A., *J. Physiol.* **101**, 200 (1942).

There is a close parallelism between voltage per centimeter and concentration of Ch. E. (Nachmansohn, Cox, Coates, Machado, 107). Both decrease in similar proportions from the head to the caudal end of the electric organ. The results of one typical experiment are reproduced in

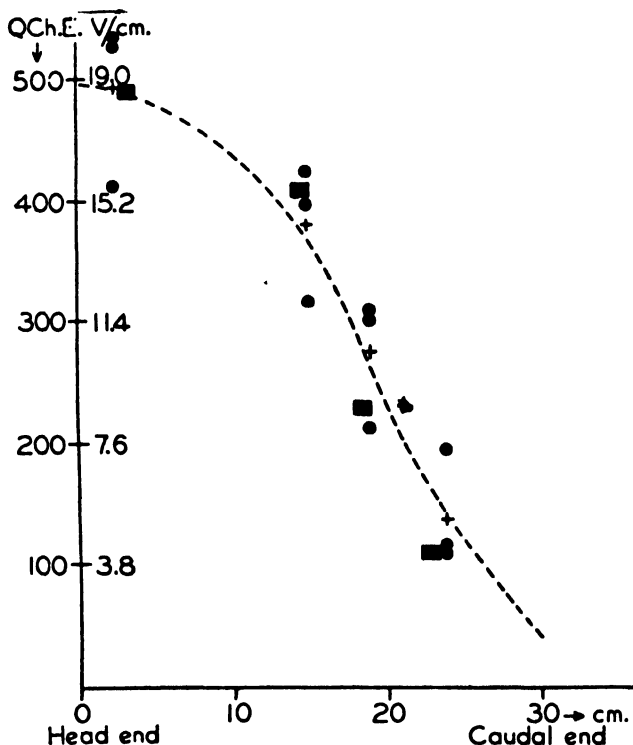


Fig. 2. Action Potential and Choline Esterase Activity in the Electric Organ Specimen No. 1. Length of fish 51 cm.

Abscissae: distance from the anterior end of the organ in cm.

Ordinates: QCh.E. and V/cm.

- average QCh.E. from a single piece of tissue.
- + average QCh.E. values from pieces of the same section.
- V/cm.

Fig. 2. Even in absolute values V per cm. and Q Ch. E. of different specimens are comparable. It is possible to predict from the Ch. E. concentration the maximum voltage within 10-15 per cent and *vice versa*. The histological preparations show that the aspect of the Voltaic pile changes at different sections and also differs according to specimen and size. This is illustrated in the photographs of Fig. 3, which are reproductions at the

same magnification of different sections of a large and a small specimen. The anatomical picture corresponds to the electrical and chemical data.

It appears likely that the enzyme is concentrated at or near the active boundary as in all nerve fibers. The question may be raised as to whether the change of Ch. E. activity from the head to the caudal end of the electric organ is just an expression of active surface. This is most probably the case. But this is by no means an objection to the conclusion that a

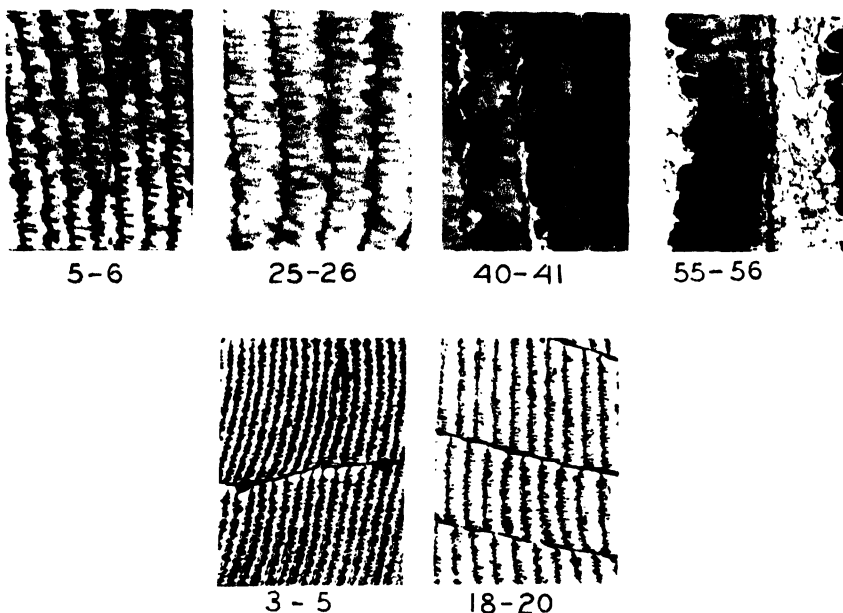


Fig. 3. Histological aspect of Electric Tissue at Different Sections of the Organ. The specimen used for the four upper sections was 114 cm. long, that used for the two lower sections was 57 cm. long. All sections are reproduced with the same magnification (145). The numbers below each section indicate the distance in cm. from the anterior end of the organ. H = Head end, C = Caudal end.

direct correlation exists between the action of ACh and the voltage of the discharge. Even if there were a concentration of Ch. E. at the surface it would not necessarily result in a quantitative parallelism between voltage of the action potential and the enzyme activity, if the two events were not intimately connected. The fact that such parallelism does exist over the wide range examined strongly supports the view that the substrate metabolized by the enzyme is intrinsically connected with the electric charge. This view gains validity from the electrogenic effect produced by



ACh injection, described on p. 355 and from the fact that the voltage produced by the injected ester is greatly increased when the removal of the ester by Ch. E. is inhibited by eserine.

The parallelism between the voltage of the action potential and the concentration of Ch. E. appears to be specific. No other compounds or enzymes tested so far showed a similar curve. In contrast to the variations of Q Ch. E., the rate of respiration is equal throughout the organ and does not differ markedly between the specimens of different size. The rate of respiration is moreover remarkably low in absolute values, the  $Q_{O_2}$  at 20–23° varying between  $-0.3$  and  $-0.5$  (Nachmansohn, Cox, Coates, Machado, 107). This is the same order of magnitude as observed in other nerve fibers at this temperature. The low rate of respiration is of particular interest if compared with the high rate of ACh metabolism. If the Q Ch. E. is 150–200, about 10,000 times more molecules of ACh can be split per unit of tissue and unit of time than molecules of oxygen can be taken up. If the  $O_2$  uptake after activity is increased to twice the observed value, the rate would still be 5000 times as high. These figures are minimum values and do not indicate the real ratio between the possible rate of ACh hydrolysis and that of oxidation. For the Q Ch. E. values are calculated per unit weight of tissue whereas it is known that ACh metabolism occurs only at or near the surface of the neuron, that is, within a minute fraction of the actual tissue weight. This correction does not have to be made for oxidation which occurs throughout the axoplasm (Nachmansohn and Steinbach, 105; Nachmansohn, Steinbach, Machado, Spiegelmann, 106). On the assumption that 90 per cent of the tissue do not contain Ch. E., the rate of ACh hydrolysis would be approximately 100,000 times as high as that of respiration, but it may be closer to one million. The figures indicate, however, only the *possible rate*, not the *absolute amounts* really metabolized. ACh is released and hydrolyzed within milliseconds. The duration of the discharge is about 3 milliseconds, the number of discharges until fatigue about 3000; the whole duration of the discharges therefore about 9 seconds. The discharges may occur within a very short time since the fish may discharge at the rate of 100 per second. The complete recovery requires more than an hour. The increased rate of respiration may continue for one to two hours. In absolute values therefore the difference between the amounts metabolized becomes small (see page 364). This is important for the understanding of the energy relations. But for the short time of the passage of the impulse the difference of rates is extremely great.

#### V. NEW CONCEPT OF THE ROLE OF ACETYLCHOLINE

The three essential facts which emerge from the observations described so far are (1) the high concentration of Ch. E. indicating a rate of ACh

metabolism sufficiently high to parallel the electrical events during nerve activity; (2) the exclusive and specific localization of Ch. E. at the neuronal surface; and (3) the parallelism between the concentration of the enzyme and the voltage of the action potential. These findings in connection with all the other known facts suggest that the release of ACh is not limited to the nerve endings but occurs everywhere at the neuronal surface and that the ester is intimately connected with the electrical changes during activity.

The mechanism of nervous conduction is generally pictured in the following way: the nerve fiber is enclosed by a polarized membrane. The polarized state of the membrane is due to a selective permeability to K ions, which are present in different concentrations on the two sides of the membrane. Any stimulus acting on the cell surface causes the local breakdown of resistance and increased permeability to all ions. Thereby the membrane becomes depolarized, the resting potential disappears or may be even reversed. A potential difference is thus established between this point and the inactive adjacent region since the depolarized point is negative to the polarized. This potential gradient causes flow of current to the depolarized region. By the flow of current the adjacent region is stimulated and in its turn depolarized. This starts the same process and thus the depolarization spreads along the fiber. Webb and Young (129) have recently shown that there is a good agreement between the action potentials observed and the diffusion potentials which would arise if the membrane were permeable only to potassium ions and were rendered permeable to anions during the passage of the impulse.

The relationship between ACh and the voltage of the action potential can be conceived in two ways. Since  $V = E - IR$  the ester may either generate an E. M. F. by action on the surface or it may decrease the resistance of the membrane. Resistance and E. M. F. are closely related properties of the membrane. Cole and Curtis (21) have shown by alternating current impedance measurements that the action potential is associated with a transient change in resistance which falls from 1000 ohm per  $\text{cm}^2$  to 25 ohm. It is conceivable that a substance which appears and disappears within milliseconds could be responsible for these changes. The depolarization may be caused by the release of ACh at the point where the stimulus reaches the surface of the membrane. Thus, flow of current is generated to this point from the adjacent regions. This flow of current acts as stimulus of the next section and releases ACh there. The same process is repeated and thus the impulse is conducted along the fiber. This idea is quite compatible with the concept of propagated impulses as developed by Keith Lucas and Adrian. The polarized state is rapidly restored by the removal of the free ester by its hydrolysis. At the nerve ending where the surface increases and therefore the resistance decreases

this process would lead to a greater flow of current, thus enabling the impulse to cross the non-conducting gap.

In the original theory, ACh liberated at the nerve ending acts as "humoral" or "neurohumoral" transmitter directly on the effector organ or on the second neuron. According to the new concept, the release of ACh is an intracellular process generating flow of current by action on the cell membrane, but the transmitting agent is the flow of current. As outlined above, the electrical signs of nerve activity do not justify the assumption of a special mechanism responsible for transmission at synapses. The new concept removes the chief difficulty in reconciling the electrical and the chemical theories of transmission of nerve impulses. It makes it unnecessary to assume a basic difference in the role of ACh for conduction along fibers and across synapses. It is fundamentally identical with the picture of the mechanism of conduction as developed by electrophysiologists. This picture was never considered as being complete (Adrian, 1, page 20). Gasser (36) comparing the spikes to the ticks of a clock points out that both are but signs of activity in an underlying mechanism:

"It follows then that if spikes are but manifestations of activity in the inherent mechanism of nerve fibers, the story of nerve is by no means told when the spikes have been described. We need to know something about the mechanism which produces them—how it is maintained, its capacity for work, and when and how the work is paid for."

The investigations reviewed in this paper add one additional point of information: that the release of ACh is an essential link in the generation of the electric changes. The picture is far from being complete: the mechanism of the release of ACh remains unknown as well as the physico-chemical changes of the surface membrane which are produced by the ester and which lead to breakdown of resistance and increased permeability. On the other hand some light has recently been thrown (see the following paragraphs) on the mechanism of formation as well as on other chemical reactions initiated by the breakdown of ACh.

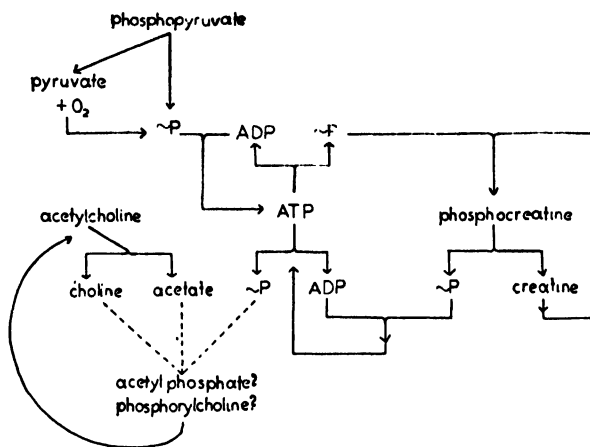
## VI. THE "ACETYLCHOLINE CYCLE"

The alterations of the nerve membrane during activity and their rapid reversal cannot be conceivably effected without energy loss. If the release of ACh and its subsequent breakdown are responsible for the alterations of the nerve membrane, chemical reactions must supply the energy for the resynthesis of ACh in the recovery process.

The most readily available source of energy for endergonic life processes is the energy of phosphate bonds. In muscle the breakdown of adenosine-triphosphate (ATP) appears to be the primary energy source of the con-

traction (Meyerhof, 85). Recent work of Engelhardt (34), of Needham (113) and of Bailey (6) has led to the idea that ATP may be directly connected with the mechanical changes during contraction. If the ATP gives off one energy-rich phosphate the remaining adenosinediphosphate is immediately rephosphorylated by the breakdown of phosphocreatine (PhCr). PhCr thus acts as a "storehouse" or "accumulator" for energy-rich phosphate bonds (Lipmann, 64).

When PhCr was found in nerve it was assumed that it may have a similar function there as in muscle and may yield the energy for the action potential (Feng, 41; Gerard, 48; Hill, 58). This possibility was tested



Description of Figure

The "acetylcholine cycle"  
 ATP = adenosinetriphosphate  
 ADP = adenosinediphosphate  
 ~P = energy rich phosphate bond

FIG. 4

repeatedly by Gerard and his associates (50-52). These investigations have shown that PhCr breaks down during prolonged nerve stimulation. In ordinary nerves, however, both electrical and chemical changes are small and it is difficult to obtain quantitative correlations. Only general estimates are possible. The electric organ of *Electrophorus electricus* offers a suitable material for correlating chemical and electrical energy released by the action potential, since both are within the range of measurement. The electric energy of the discharge in a section of the electric organ has been determined and compared with the energy released by the breakdown of PhCr measured simultaneously (Nachmansohn, Cox, Coates, Machado, 108).

The electric energy released externally per gram and impulse was found to be (as an average of 15 experiments), about  $8 \times 10^{-6}$  gcal., with an external resistance which—as controlled by a cathode ray oscillograph—yields the maximum external energy. The variations between different experiments were small, the values varying usually between 5 and  $10 \times 10^{-6}$  gcal. The total electric energy was found to be (as an average of 6 experiments), about  $48 \times 10^{-6}$  gcal. (Cox, Coates, Brown, 25). The specimens available were all of similar size and the section measured was always near the head end. Values obtained on other sections and on specimens of different size may differ. The breakdown of PhCr, measured simultaneously with the electric recordings on the same section, yielded per gram and impulse on the average  $32 \times 10^{-6}$  gcal., with variations ranging generally from 20 to  $50 \times 10^{-6}$  gcal. This amounts to about four times the electric energy released externally. The calculations are based on the assumption that the free energy of one mole of PhCr is 10,000 gcal.

In muscle, one part of the creatine is rephosphorylated by oxidation energy. This is a slow process. Another part is immediately rephosphorylated by phosphopyruvic acid, an intermediate of lactic acid formation, which transfers its phosphate to creatine, the ATP acting again as intermediate link ("Parnas reaction"). The first investigations on a possible formation of lactic acid connected with nerve activity were either negative or doubtful. After prolonged stimulation a slight rise of lactic acid was found in frog's sciatic (5 mg. per cent), but none in rabbit's nerve (Holmes, Gerard, Solomon, 61). Referred to the energy required the amount of 5 mg. per cent, if correct, is quite considerable. With the chemical method at that time used it was close to the limit of possible measurement. From experiments on nerves poisoned with iodoacetic acid, Feng (40) concluded, in 1932, that formation of lactic acid, although not essential to nerve activity, is required if the nerve is to endure prolonged activity in oxygen. If the nerve was poisoned with iodoacetate, its capacity for prolonged activity in oxygen was improved by addition of sodium lactate. These observations were confirmed by Chang and Gerard (18).

The formation of lactic acid as result of the action potential has been demonstrated on the electric organ (Nachmansohn, Cox, Coates, Machado, 108). 150  $\mu$ g. of lactic acid formed were found to be formed (as average of a series of experiments) after 1600 impulses. On the assumption that the formation of one mole of lactic acid releases 16000 gcal. without inclusion of heat of neutralization, the energy supplied by the lactic acid formed is on the average  $16 \times 10^{-6}$  gcal. per gram and impulse. It is probable that this energy is used as in muscle, to rebuild immediately part of the PhCr split and that we measure only the balance between PhCr actually split and rebuilt. The energy supplied by these two chemical reactions measured

amounts to about  $45\text{--}50 \times 10^{-6}$  gcal. per gram and impulse. The energy released by these two chemical reactions thus equals the total electric energy released.

These results offer evidence that the breakdown of PhCr is adequate to account for the electric energy released by the action potential. Hence it appears probable that the phosphate bonds of this compound are the energy source for the restoration of the resting condition of the membrane after the passing of the nerve impulse, ATP acting as intermediate link, as in muscle. They are consistent with the conclusion that phosphate bonds are used for the resynthesis of ACh.

The amount of ACh actually released during the action potential of the electric organ is not known. An indication of the quantity involved may be obtained from an estimation of the amount which can be split during the duration of a discharge if we assume that ACh is released during that period at a rate which is of similar order of magnitude to that at which it can be split. The duration of a single discharge is about 3 milliseconds. One gram of electric tissue can split about 1  $\mu$ g. of ACh during that period, that is  $5 \times 10^{-6}$  millimoles. But it is not probable that this whole amount is actually released since it is reasonable to assume that the enzyme which has to remove the active compound is present in excess. The amount of PhCr split per gram and impulse is about 0.7  $\mu$ g. or  $3 \times 10^{-6}$  millimoles. Thus the amounts of ACh and PhCr metabolized per gram and impulse seem to be of the same order of magnitude even if we assume that Ch. E. is present in a concentration of about 2-5 times in excess of that necessary to remove the ACh released at a sufficiently high rate. This makes it appear probable that acetylation of choline occurs by means of an exothermic phosphorylation. Evidence for this assumption has been subsequently offered and formation of ACh in presence of ATP has been demonstrated (see following chapter). The intermediate steps have not been established as yet. One possibility is the formation of acetylphosphate, the compound described by Lipmann (65). If each energy-rich phosphate bond were used only for the formation of one molecule of ACh there would arise the following difficulty: the synthesis of an ester like ACh probably requires about 1500-2000 gcal. per mole, which represents only one fifth to one seventh of the energy released by one mole of PhCr. Since the energy of the breakdown of PhCr equals approximately the total electric energy released, apparently no loss of energy yielded by the phosphate bonds occurs. The question is how the remaining energy is used. The phosphate may act simultaneously on a protein in a way similar to that conceived by Kalckar (62) for the interaction between myosin and adenosinetriphosphate. But there are other possibilities. No information is available at present and the problem requires further investigation.

But the principal steps can be pictured in the following way: ACh released by the impulse is split into choline and acetic acid. It is resynthesized with the free energy of ATP which gives off one energy-rich phosphate. The remaining adenosinediphosphate is rephosphorylated by the phosphate of the PhCr. The creatine is rephosphorylated immediately, partially from phosphopyruvic acid, ATP acting again as intermediate, and partially during the recovery, by the energy of pyruvic acid (or carbohydrate) oxidation. Since this whole chain of reactions connected with the nerve action potential is initiated by the release of ACh, it has been called the "acetylcholine cycle."

The great difference between the rate of ACh metabolism and that of respiration has been discussed previously (page 358). It appears of equal interest to compare the absolute amounts metabolized. Since the amount of PhCr split appears to be, as discussed above, of the same order of magnitude as the amounts of ACh split, it may be used as basis for such estimation.

$5.2 \times 10^{-6}$  moles PhCr are split per gram electric tissue and per 1600 impulses.  $1.5 \times 10^{-6}$  moles  $O_2$  are taken up per gram and hour assuming a  $Q_{O_2}$  of  $-0.4$ . According to Ochoa (115) for one atom O used three energy-rich phosphate bonds may be produced in heart extracts (*i.e.*, P:O ratio of 3). In electric tissue the P:O ratio has not been measured but it is reasonable to assume that it is of the same order of magnitude, so that  $0.86 \times 10^{-6}$  moles  $O_2$  would be sufficient to regenerate the phosphate bond energy lost.

The phosphate bond energy corresponds to only two-thirds of the total energy released. The total energy released per gram electric tissue and 1600 impulses was found to be  $8 \times 10^{-2}$  gcal. 1 g. of electric tissue takes up 32 cmm.  $O_2$  in 60 min., calculated again for a  $Q_{O_2}$  of  $-0.4$ . Assuming  $4.8 \times 10^{-3}$  gcal. for one cmm.  $O_2$  metabolized in the cell, 32 cmm. would amount to  $15 \times 10^{-2}$  gcal. The efficiency of the conversion of oxidation energy to phosphate bond energy is nearly 60 per cent (Ochoa, 115). Thus  $13 \times 10^{-2}$  gcal. of respiration energy would be required to account for the energy lost by PhCr breakdown and lactic acid formation. The  $Q_{O_2}$  was determined on tissue slices and may be higher than the actual resting metabolism, although probably not as high as that following activity. Even if we assume an oxidation energy of  $10 \times 10^{-2}$  gcal. in the resting condition, a two-fold increase over a period of 80 min. would account for the energy required. This is well within the range of the increase of respiration found in nerves after activity.

The heat production of the discharge in the electric organ has not yet been measured. But since the discharge is considered as being fundamentally identical with the ordinary nerve action potential, it is interesting to compare the chemical data with the heat production measured by A. V. Hill

(58) and his associates on nerves. The total heat production in the crab nerve per gram and impulse is about  $35 \times 10^{-6}$  gcal. In frog's nerve it is about  $4-5 \times 10^{-6}$  gcal. at  $0^\circ \text{C}$ . and  $10^{-6}$  gcal. at  $20^\circ \text{C}$ . In the electric organ, PhCr breakdown and lactic acid formation yield about  $45-50 \times 10^{-6}$  gcal., to which the oxidation heat has still to be added. But it is obvious that the order of magnitude is the same. Minor differences are always possible even if the process is fundamentally identical. The PhCr content of nerve is lower than that of the electric organ, although referred to the active surface the difference may be small. It has to be kept in mind that in the electric discs the neuronal surface is quite considerable, probably even more extensive than that of nerve endings of motor end plates.

For an appreciation of the role of PhCr as energy source of the electric energy, it is useful to compare the concentration in the electric organ with that in muscle. The electric organ contains about 5-7 mg. of PhCr per gram. The total dry matter amounts to 80 mg. per gram. Consequently about 6-9% of the total dry matter is PhCr. In frog muscle the amount of PhCr is about 4-5 mg. per gram. This is about 2 per cent only of the total dry matter since the dry matter of muscle is three times as high as that of the electric organ. According to a calculation of A. V. Hill, a single nerve impulse requires only one fifty-thousandth of the energy of a muscle twitch. Although the energy per gram and impulse in the electric organ is, as just pointed out, higher than that observed in ordinary nerve fibers, it is still of the same order of magnitude. The higher concentration of such an energy-rich compound as PhCr in an organ, requiring for its activity so much less energy than the muscle, is in itself suggestive of the important role of PhCr as energy source of the action potential.

#### VII. THE FORMATION OF ACETYLCHOLINE; CHOLINE ACETYLASE

The conclusion that phosphate bond energy is used for the formation of ACh has been tested experimentally. ACh formation was already observed by Quastel and his colleagues (76). These authors demonstrated that ACh is formed in brain slices, *i.e.*, in intact cells, but under *aerobic* conditions only and if glucose or pyruvate is present. In no other tissue was ACh synthesis found. The finer mechanism of the formation was not elucidated.

For the analysis of enzyme mechanisms cell-free extracts are preferable. In agreement with the conclusion above an enzyme, choline acetylase, has been extracted from brain which in the presence of ATP, in a cell-free solution, and under *anaerobic* conditions synthesizes ACh (Nachmansohn and Machado, 109). Only nervous tissue seems to contain the enzyme. Kidney, liver, and muscle do not contain measurable amounts. Extracts prepared from one gram of rat or of guinea pig brain form 120-160  $\mu\text{g}$ . of ACh in 60 min. (Nachmansohn and John, 111). Extracts from cat and



pigeon brain seem to be slightly less active. The enzyme loses its activity rapidly at 37° C.; even if kept in the refrigerator it remains stable for a few hours only. It is not sensitive to changes of pH close to pH 7 (between 6.6 and 7.4).

Ochoa has shown that fluoride inhibits the activity of adenosinetriphosphatase without interfering with the transfer of phosphate from ATP to a phosphate acceptor (114). This may be due to the precipitation of Ca since it is known that calcium activates adenosinetriphosphatase (Bailey, 6) whereas magnesium inhibits it (Greville and Lehmann, 56). Addition of fluoride greatly increases ACh formation. Addition of choline is necessary whereas that of acetate has, under the conditions used, practically no effect. Eserine has to be present to inactivate choline esterase.

Whereas in the first experiments no marked difference was found between concentration of  $K^+$  between 0.02 and 0.06 *M*, it could later be established that  $K^+$  is necessary for the activity of the enzyme: the activity increases considerably if the concentration of  $K^+$  rises (Nachmansohn and John, 111). Still more marked is the influence of  $K^+$  in the dialyzed enzyme, in which a strong reactivation is only possible in presence of  $K^+$  concentrations which are of the same order of magnitude as found inside the nerve cell.

Barron has recently emphasized the significance of sulfhydryl groups, present in many enzyme proteins (9). Formation of ACh is strongly inhibited by iodoacetate which, as shown by Rapkine (121, 122) and Dickens (30), reacts specifically with —SH groups. Iodine, which easily oxidizes —SH groups, has also a marked inhibitory effect at  $1 \times 10^{-4}$  *M* concentration if allowed to act on the enzyme for 40–60 min. Sulfhydryl groups are highly sensitive to copper although this effect is not specific. Formation of ACh was practically completely inhibited by a  $3 \times 10^{-5}$  *M* concentration of copper ions, and the inhibition was still 66 per cent at a  $1.5 \times 10^{-5}$  *M* concentration. Thus it appears that choline acetylase belongs to the large group of enzymes in which the protein contains —SH groups (Nachmansohn and Machado, 109). Further support for this assumption may be seen in the observation that cysteine and cyanide increase the enzyme activity (Nachmansohn and John, 111).

On dialysis, the activity of choline acetylase decreases rapidly. After two hours of dialysis the enzyme loses about 80 to 85 per cent of its activity. Clinical observations suggest that glutamic acid has a favorable effect on epileptic patients suffering from *petit mal* attacks, whereas patients with *grand mal* do not appear to be affected (Price, Waelsch, Putnam, 120). On the hypothesis that the slow waves which appear in the electroencephalogram during attacks of *petit mal* may in some way be connected with a lower rate of formation of ACh, the effect of glutamic acid has been tested on the synthesis of ACh in dialyzed extracts of rat brain.

Addition of glutamic acid in  $2 \times 10^{-2} M$  concentration to extracts dialyzed for two hours increases the rate of formation of ACh. Only the natural *l*(+)-glutamic acid has a strong effect; *d*(-)-glutamic acid has a small effect (Nachmansohn, John, Waelsch, 110). With  $K^+$  and glutamic acid about 50–80 per cent of the original activity may be regained. With decreasing concentrations of glutamic acid the effect decreases, but is still present at a concentration of  $1/800 M$ . The concentration of glutamic acid found in the brain by Cohen (20) is of the same order of magnitude.

Of all other amino acids tested, only cysteine has a still stronger effect. With  $K$  and cysteine nearly a complete reactivation may sometimes be obtained. *l*(+)-Alanine has a weaker but still marked effect whereas all other amino acids have either none or very weak effects (Nachmansohn and John, 111). Of other compounds tested so far only citric acid was able to reactivate markedly the dialyzed enzyme. The effect is nearly as strong as that obtained with *l*(+)-glutamic acid.

The mode of action of these compounds has still to be explained. But the action of these naturally occurring compounds on ACh formation appears of theoretical as well as of clinical interest in view of the effect observed with one of them in disorders of nervous function.

Choline acetylase may also be extracted from powder of acetone-dried brain. In these preparations choline esterase is destroyed to a large extent. Sometimes the destruction of choline esterase is nearly complete. In this way the two enzymes may be separated (Nachmansohn and John, 111).

Another question yet to be answered is the role of vitamin  $B_1$  in the mechanism of ACh formation. Oxidation of pyruvic acid may sometimes go through formation of acetic acid (Long, 70). Acetylation of various substances occurs easily subsequent to pyruvic acid oxidation. It therefore appears possible that the acetyl unit of ACh is derived from pyruvic acid. The observation of Quastel and his colleagues that presence of pyruvic acid (or glucose) is necessary for the formation of ACh in brain slices in the aerobic condition is, however, not conclusive since in these conditions oxidation of pyruvic acid may be the source of energy supply. Peters (117) has shown that oxidation of pyruvic acid requires vitamin  $B_1$ . The active form is diphosphothiamin (cocarboxylase) (Ochoa and Peters, 116; Banga, Ochoa, Peters, 7). Mann and Quastel (75) investigated whether or not the formation of ACh in brain slices, kept in pyruvate—Locke—bicarbonate media, is accelerated in presence of vitamin  $B_1$ . The vitamin had no effect in brain of normal pigeons, but in vitamin-deficient pigeons addition of vitamin  $B_1$  definitely increased the rate of ACh formation, although only in presence of a high potassium concentration. But this finding too may be explained in terms of increased energy supply. Nachmansohn and Steinbach (105) found in experiments on the giant axon of the squid that the concentration of vitamin  $B_1$  (determined as diphosphothiamin) is con-

siderably higher at the surface of the axon than in the axoplasm, although the localization is not as exclusive as that of Ch. E. Since there is a high rate of ACh metabolism one possible explanation may be that pyruvate is metabolized at a higher rate there. However, pyruvate is an intermediate for many reactions and the vitamin may still have another function.

## VIII. DISCUSSION AND SUMMARY

### 1. Discussion

Since the picture obtained with the new approach differs from the original concept, it appears of interest to analyze some of the previous methods used. This may be helpful for the understanding of the problem involved.

The basis of Elliot's suggestion as well as of the concept of Loewi and Dale is the similarity between the action of drugs or active chemical compounds, and the effect of nerve stimulation. Such a similarity, however, does not imply that the mechanism of these actions is identical. A nerve or muscle cell may be stimulated by many different kinds of stimuli: electric, chemical, thermal, etc. We cannot conclude from the effect alone that the stimulus which has an exciting effect is identical with the physiological stimulus. The picture became complicated when a naturally occurring compound was found which appeared to be actually released from the nerve and had a stimulating effect.

It is true that ACh is a compound which may have under certain conditions a stimulating effect. The question is, however, whether in the physiological event of nerve transmission ACh really leaves the nerve ending and acts on the effector cell directly or whether its action is intracellular, namely depolarization of the nerve membrane and generation of current as maintained in the new concept. The appearance of ACh in the perfusion fluid does not yet indicate anything about its mode of action. Many compounds of intermediate cell metabolism are known which under certain conditions may appear outside the cell. Even if the release of ACh were an intracellular process directly connected with the action potential, a fraction of the ester might pass into the perfusion fluid. As with all enzymatic processes, the curve of hydrolysis is not straight but logarithmic. Part of the ACh released may therefore persist for a longer time than the short interval of the refractory period. These amounts, varying according to different cells and their condition, may leak out and appear in the perfusion fluid. The statement that the concentration of Ch. E. is sufficiently high to remove the ACh released within the refractory period, means that more than 50 per cent of the ester released can be removed during this time ("halftime value").

If, however, the assumption of a direct action on the effector cell is correct one should expect the amount which appears in the perfusion fluid to be of the same order of magnitude as that which has a stimulating effect. But there is, on the contrary, a great discrepancy between the amounts of ACh collected in the perfusion fluid and the amount which has a stimulating effect. In the superior cervical ganglion, the amount of ACh which stimulates the ganglion in the presence of eserine is about  $1\text{ }\mu\text{g.}$ , the threshold dose being about  $0.1\text{ }\mu\text{g.}$  (Feldberg and Vartiainen, 38). According to the figures of MacIntosh (74), considered as being the most reliable, the amount collected per single maximal shock is  $0.000015\text{--}0.000035\text{ }\mu\text{g.}$  of ACh, the mean value of 24 observations being  $0.000024\text{ }\mu\text{g.}$  This is only 0.024 per cent of the amount necessary to produce a minimal and 0.0024 per cent of that necessary to produce a full response. The material and experimental conditions in this case are relatively favorable. In the case of striated muscle the discrepancy is even more conspicuous: the amount necessary to produce a maximal twitch was found to be 100,000 times as high as that liberated per nerve stimulus (Brown, 16) as compared with 40,000 times in the sympathetic ganglion.

It is difficult to reproduce the effect of nerve stimulation even with the best technique such as that used, in which the ester was injected rapidly, in a very small volume and at close range. It is therefore reasonable to expect that the amount necessary for producing a stimulating effect would be higher than that actually released. In their publication on the transmitter function of ACh in striated muscle, Dale and his associates gave by mistake a figure in which the difference was only 100 times (17). In the discussion they pointed out that such a difference may be explained by the difficulty of reproducing the physiological event. But for the corrected figure of 100,000 times such an explanation appears less satisfactory. Moreover, the preparations were perfused by Ringer or Locke solution containing eserine. As we know, a high concentration of Ch. E. is present at synapses and end plates outside as well as inside the nerve endings. The enzyme present outside forms a powerful barrier for the ACh diffusing from the cell. This mechanism, which should prevent the presence of ACh outside the cell, is in the experimental condition inhibited. If even then the discrepancy is of such order of magnitude as that which was found, it appears doubtful whether under normal conditions, *i.e.*, in the presence of Ch. E. an amount of ACh necessary for direct stimulation would be able to diffuse across the neuromuscular junction or synapse to the effector cell in sufficiently high concentration.

It may be noticed that the absolute *amounts* are discussed and not the concentrations. We do not know the actual concentration. Although the concentration may be high, it remains true that the difference between

the amounts collected and the amounts used for stimulation appears too great for a direct action to be possible. It may be difficult to collect the total amount, but the loss should be small if eserine is added and the ACh released actually does leave the cell. The amounts are also small if compared with those which may be metabolized. Here we meet with another difficulty for the theory of an extracellular action. If ACh acts only after having left the cell, the question arises as to why the enzyme is so highly concentrated *inside* the nerve fiber. In the case of the superior cervical ganglion as much as 60 per cent is localized inside the pre-ganglionic fibers. Such a distribution is consistent only with the assumption of an *intracellular* process. It explains also why the amounts which appear outside the cell are small. From the concentration of Ch. E. inside the pre-ganglionic fibers it was calculated that 1-2 per cent of the enzyme present would be sufficient for the removal of ACh within one millisecond. This would represent a very small fraction of the possible rate in view of the high concentration of the enzyme. The excess of enzyme would be still greater in the case of muscle. But if we assume that most of the ACh released acts inside and is removed there and only that fraction diffuses which escapes hydrolysis inside the cell, then the amounts found in the perfusion fluid are well within the range which we may expect from the enzyme concentration present.

The appearance of ACh in the perfusion fluid was considered as being one of the fundamental facts in favor of the assumption of a direct action. The figures discussed, considered by themselves, may not exclude it. But combined with all the other objections and difficulties they form a serious obstacle, whereas they appear consistent with the idea of an intracellular process in which only a part of the ACh released leaves the cell. This interpretation also removes the difficulty encountered by the assumption that the enzyme, highly concentrated everywhere at the neuronal surface, should suddenly have at the nerve ending a function entirely different from that which it has everywhere else in the axon.

Another important factor which has to be considered is the condition of the cell. Loewi's experiments have been repeatedly criticized. Asher claimed that he could reproduce Loewi's experiment on the frog heart only in hypodynamic hearts (2, 112). If the heart was in good condition, the perfusion fluid did not contain the active compound. The term "hypodynamic" may be open to discussion, but in a heart perfused with Ringer solution instead of blood, the active surface after a number of stimuli may no longer be in an entirely physiological condition. Under such conditions more ACh may diffuse into the surrounding medium than in a normal heart. It may be recalled that Kibjakow, who first demonstrated the liberation of ACh in the perfused ganglion and suggested that the ester may be the

chemical transmitter from neuron to neuron, did not use eserine. Feldberg and Gaddum were not able to reproduce his experiments unless eserine was added to the perfusion fluid (37). This may well be due to the fact that Kibjakow's preparation was in poorer condition than that of Feldberg and Gaddum and that therefore the amount of ACh which leaked out in his experiments was just sufficiently high to be detected. Eserine is a strong drug, and we are altering the physiological conditions by its administration.

Whatever experimental method is applied we no longer have a strictly normal condition. There is always a question, therefore, as to whether such far-reaching conclusions may be drawn concerning the physiological mechanisms from the facts observed in an experiment. The fact that ACh appears after nerve stimulation—and only then—indicates that this compound is a product of nerve activity. But the original interpretation of the mechanism of its physiological role is based on very special conditions, and the idea that the ester is secreted from the nerve ending and acts directly on the second neuron or on the effector cell is difficult to reconcile with the many known physiological, electrical, and biochemical facts. It would, moreover, require an explanation as to how this "direct action" on the effector cell should be pictured. The term "neurohumoral" or "synaptic" transmitter gives no indication of the mechanism of its action.

Besides the response of the effector cell to ACh and the appearance of ACh in the perfusion fluid after nerve stimulation, a third effect was considered necessary to establish the "cholinergic" nature of a nerve ending: addition of eserine had to have a potentiating effect.

In view of the high affinity of eserine for Ch. E., it appears almost certain that the potentiating effect of eserine can be attributed to the inhibition of Ch. E. and the persistence of ACh. But this does not justify the conclusion that if eserine has no potentiating effect ACh is not involved. Drug effects are extremely complex. Affinity for an enzyme is one of the many factors involved, but the effect depends on many others. One of the most important is certainly the permeability of the cell membrane to a drug. Selective permeability has always been considered one of the fundamental functions of the cell membrane. Much work has been done on this problem in recent years and considerable progress has been achieved (see for instance the *Cold Spring Harbor Symposium* in 1940). "Cell permeability has passed from the qualitative to the quantitative stage," writes E. Newton Harvey in the Foreword to Davson and Danielli's book on "Permeability of Natural Membranes" (29).

It appears surprising that the question of permeability has found relatively little consideration in the problem of the action of eserine and

prostigmine. Eserine is a tertiary ammonium salt, prostigmine a quaternary ammonium salt. Except for the fact that the quaternary form is not soluble in lipoids, very little is known about the conditions under which drugs enter the cell and no data are available about the permeability of the nerve membrane to these drugs. Some of them enter the living cell, others not. Varying fractions may enter under different conditions and in different tissues. Thus the impermeability of the cell membrane to eserine may be an essential factor in preventing the effect of this drug in many instances. Other factors may be of importance, *e.g.*, the concentration, which depends on the rate at which the drug is carried to the site of action by the circulation as compared with the rate of its removal either by enzymatic destruction or by circulation. Other chemical compounds, present in some cells and lacking in others, may react with the drug. All these factors make the interpretation of drug effects extremely difficult. In such complex systems as cells or organs the effect of a drug upon an enzyme reaction permits of interpretation only when an action is observed. The failure of the drug to act is less significant and in most cases difficult to explain. Even in a homogeneous system only positive effects permit conclusions. If an effect of carbon monoxide is found in an enzyme solution, this probably indicates that the enzyme contains an active metal. But if carbon monoxide has no effect, it cannot be concluded that no metal is involved. Considering the fact that eserine under certain conditions does not potentiate the effect of nerve stimulation, Dale (28) suggests that ACh may be removed by some other mechanism than by Ch. E. This assumption raises many other difficulties (Nachmansohn, 98). It seems less difficult to consider that ACh is inactivated by the specific enzyme system which fulfills quantitatively the postulates required, even though the potentiating effect of eserine cannot be demonstrated under all conditions. Assuming that the effect of eserine is due to the inhibition of Ch. E., the answer to the question, what the finer mechanism may be and under what conditions it acts, is unknown.

Similar considerations may be applied to the effect of strychnine. As shown by Nachmansohn (93, 94), this alkaloid has a high affinity for Ch. E. If only a small fraction of the amount necessary to produce strychnine convulsions in the frog reaches the synapses, it would be sufficient to decrease markedly the Ch. E. activity there. The effect produced as well as the increased amount of ACh found in the brain in strychnine intoxication may therefore be attributed to inhibition of Ch. E. But it remains an open question as to why strychnine, in contrast to eserine, affects only the central nervous system and even there has some centers of preference, whereas it does not effect the periphery.

In connection with the variety of the effects of a drug, another aspect

of the problem may be mentioned. According to the theory presented here, ACh has the same function in the transmission of impulses of all nerves, whether afferent or efferent, peripheral or central, sympathetic or parasympathetic. Ch. E. is highly concentrated in all these nerve cells and it is difficult to believe that its significance changes. Why in some cases liberation of ACh or the response to the ester or eserine may be demonstrated and in others not, may be attributed to the many accessory factors discussed above, which at present are beyond our control.

In view of the complex nature of biological phenomena no single factor like the localization or concentration of Ch. E. would be sufficient to support a theory concerning the role of ACh in the mechanism of nervous action. But the combination of so many facts as are presented here lends additional weight to each of them and supports strongly the interpretation given above. The concept of the mechanism of nerve activity suggested is far from being complete, as emphasized before, and will require correction when new facts are presented, but at present it appears to be the best interpretation of the great number of facts available.

#### *4. Summary*

1. The following concept of the role of acetylcholine in the mechanism of nervous action is suggested: the ester is released at the neuronal surface when a stimulus reaches the nerve cell. By the action of ACh the permeability of the membrane to ions is increased and hence a depolarization occurs. The depolarized point becomes negative to the adjacent region and flow of current is hereby generated. This flow of current transmits the impulse to the following point, where again ACh is released and the process repeated. In this way the impulse is propagated. The transmitting agent is the flow of current, but the current is generated by the release of ACh. The idea is consistent with the concept of propagation of nerve impulses as proposed by Keith Lucas and Adrian.

The role of ACh is the same in the transmission of the nerve impulse along the axon and across the synapse. There is only a quantitative difference: due to the increased surface at nerve endings, there is less resistance and therefore more flow of current enabling the impulse to cross the non-conducting gap.

2. The concept is based mainly on three findings: (i) on the rate of ACh metabolism which as indicated by the concentration of choline esterase, is sufficiently high to parallel that of the electric changes; (ii) on the localization and high concentration of Ch. E. everywhere at the neuronal surface; and (iii) on the parallelism between the voltage of the action potential and the activity of Ch. E. demonstrated in the electric organ.

3. The energy released by the breakdown of phosphocreatine is adequate



to account for the electric energy released by the action potential. This suggests that the energy of phosphate bonds is used for the restoration of the resting condition of the nerve membrane. Adenosine triphosphate may act, as in muscle, as an intermediate link. Lactic acid is also formed, although in relatively lesser amounts than in muscle. Since the whole chain of reactions connected with the action potential is initiated by the release of ACh it has been called the "acetylcholine cycle."

4. An enzyme, choline acetylase, has been extracted from brain which in presence of adenosine triphosphate and under anaerobic conditions forms ACh. Some properties of the newly discovered enzyme are described.

5. Some of the methods which led to the original concept of ACh as a "chemical transmitter" are discussed in order to find a possible explanation for the difference in interpretation as result of the difference in approach.

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